DISEASE RESISTANCE AND PATHOGENIC VARIABILITY IN THE FUSIFORM RUST-SLASH PINE PATHOSYSTEM

By

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To my husband Uilson Lopes,

for his love, help, support, encouragement, and friendship.

To my family,

Deraldo, my dad (in memory);

Jandyra, my mom;

Alzira, my grandmother; and

My sister and brother;

for their love and encouragement.

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Abstract of Dissertation Presented to the Graduate School
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DISEASE RESISTANCE AND PATHOGENIC VARIABILITY IN THE FUSIFORM RUST-SLASH PINE PATHOSYSTEM

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Artificial inoculation with isolates of *Cronartium quercuum* f. sp. *fusiforme* collected from field studies were used to study temporal (yearly) and spatial (geographical) variation in fusiforme rust incidences on progress of resistant and susceptible open-pollinated of slash pine, *Pinus elliottii* var. *elliottii*. The temporal study using inoculum from six-gall mixtures of aeciospores from susceptible families showed statistically significant family, family by isolates, and family by year effects, indicating that pathogenic variability in *C. quercuum* f. sp. *fusiforme* varied among inocula from different time periods. The spatial study using single-gall isolates from families from each of four locations (family- and location-isolates) showed statistically significant effects of isolate, family, family by isolate, and family by family-isolate by location-isolate, indicating that there was pathogenic variability among single-gall isolates.

Observations on spermatial and aecial sporulation showed that statistically significant difference occurred between large and short galls. The pathogen sporulated less frequently on short galls and had fewer spores per gall surface area than on large galls, suggesting that short galls are a component of partial resistance.

Histological examination of smooth, rough and short galls (resistant reactions) demonstrated several patterns of colonization. The common resistant mechanism among the gall types was the presence of a reaction zone. However, the size, rate of development, and intensity of that reaction zone were the distinguishing features among the gall types. The pattern of colonization demonstrates different mechanisms of resistance of slash pine families to *C. quercuum* f. sp. *fusiforme.*

INTRODUCTION

Fusiform rust is the most damaging disease on slash pine in the southeastern United States. The disease significantly affects growth, causes deformities of the stem, increases management costs and, sometimes, results in complete loss of plantations. Many silvicultural options are available to forest land managers for reducing rust losses. The most economically feasible way to reduce losses caused by fusiform rust is to select and breed for host resistance. However, breeding for fusiform rust resistance is difficult due to variation in the fungus in space and in time.

In 1987, Rust Virulence field trials to characterize the spatial and temporal variability in *Cronartium quercuum* (Berk.) Miyabe ex. Shirai f. sp. *fusiforme* were established in the Coastal Plain of the southeastern United States. Slash (*Pinus elliottii* var. *elliottii*) and loblolly pine (*Pinus taeda*) progenies of rust-susceptible and rust-resistant parents were planted in 1987, 1989, 1991 and 1993. Each planting year (plantation) was a completely randomized design with five replications (blocks) of 10-tree-row plots per family.

Analyses of the cumulative rust incidence at 5 years of age for slash pine planted in 1987 and 1989 plantations showed statistically significant family by location and family by year interactions for rust incidence. This host-pathogen interaction constitutes one of the potential problems in rust resistance breeding in plants and one possible cause of break down of genetic resistance, which results from an increase in frequency of virulent types of the pathogen. It is important to find the causes of interactions, because it will have profound implications for breeding and deployment strategies, and future research in resistance. However, the cause of this interaction cannot be conclusively determined from the Rust Virulent field studies. Cumulative rust incidences on 5-year-old pine trees may have been affected by many uncontrolled factors, such as soil types, oak abundance, weather, host phenology, inoculum potential, and quantity or quality of pathotypes. The role of pathogenic variability can be better defined by standardized by artificial inoculations at the Resistance Screening Center.

The general objective of this research was to study the effect of isolates from different years and different locations (collected from the Rust Virulence studies) on rust incidences, using artificial inoculation under greenhouse conditions.

Slash pine seedlings of known rust resistance and susceptibility were artificially inoculated with fungal isolates from several locations and time periods. In one test, a selection of isolates from two time periods were evaluated to determine if there were genotype by year interactions in rust incidences, such as those occurring in the field (Chapter 2). In the second test, interactions among slash pine families and various isolates of *C. quercuum* f.sp. *fusiforme* from several locations were evaluated (Chapter 3). Inoculations were conducted at the Rust Screening Center in Asheville, NC.

Additional research investigated: (1) the ability of isolates to sporulate on slash pine families expressing susceptible (large gall) and resistant (short gall) symptoms (Chapter 4); and finally (2) histology of the infection processes and patterns of colonization by the fungus was studied in slash pine families expressing the resistant symptom- types rough, smooth and short galls (Chapter 5).

CHAPTER 1
LITERATURE REVIEW

Fusiform rust, a native disease on native hosts, was rare before 1900 (Czabator 1971) and quite slow until 1960-65 (Griggs and Schmidt 1977). From 1900 to the 1940s, ecosystem diversity resulted in a balanced ecosystem buffered against epidemics (Dinus 1974; Schmidt 1978). In the1940s forest management in the south became more intensive (Dinus 1974; Powers 1984). A vast expansion in production of seedlings in nurseries, wide-spread planting of loblolly and slash pines, improved fire protection, and intensive site preparation led to rapid growth in planted stands and increased the rust incidences to an epidemic that continues to the present. By 1950, fusiform rust was becoming a problem, and it has continued to increase in prevalence and distribution. By 1970, the disease was a serious threat to slash plantations in Florida and Georgia. During this time, many foresters began to realize that intensive forestry that promised the highest return on investment was becoming unrealistic in certain areas of the South because rust had become so serious that in some high-hazard areas it was impossible to economically manage the disease (Anderson et al. 1984). County averages in 1974 revealed that 31% of the trees were diseased, and more than one fourth of the counties sampled had greater than 50% of the trees diseased (Schmidt et al. 1974). Rust surveys showed more than 800 million slash and loblolly pine trees in South Carolina, Georgia, and Florida had potentially lethal stem infections (Powers et al. 1975). The potential benefits that could occur if rust was eliminated could range from $614 million to 4.6 billion (Pye et al. 1995).

Biology

Cronartium quercuum f. sp. *fusiforme,* a heteroecious fungus requiring both pines and oaks to complete its life cycle (Bursdall and Snow 1977; Czabator 1971), is a macrocyclic rust with five spore forms: basidiospores, pycniospores, aeciospores, urediospores, and teliospores. The life cycle of the pathogen is completed in 2 years (Czabator 1971).

Basidiospores initiate new infections by penetrating, either directly or through stomata (Miller and Cowling 1977; Patton and Johnson 1966), needles and tender young shoots of pine in late spring and early summer (March-June). The mycelium overwinters in infected pines. Hyphae grow into the stem and branch tissues, resulting in hypertrophy, hyperplasia, and the formation of the characteristic fusiform galls on pine stems and branches in 3 to 9 months (Dwinell 1977). Colonization is perennial and may persist for many years (Powers et al. 1981). In the fall, by October-December, and 2 years after inoculation, spermatia are produced in cavities under the epidermal layer of the galls (Dwinell 1977). At maturity, spermatia are exuded in viscid orange-red drops (Czabator 1971). The function of spermatia has not been demonstrated for *C. quercuum* f. sp. *fusiforme*; presumably they function as spermatia in hyphal dikariotization (Dwinell 1977; Powers et al. 1981). In spring, by February-April, aecia are produced in abundance in the active galls. Aeciospores do not require a period of dormancy and can germinate when released from the aecia. Aeciospores are wind-disseminated to oaks, where they produce a germ tube, penetrate through leaf stomata, and spread vertically and centrifugally in leaf tissues from the point of entry (Eleuterius 1968). Mycelium in the oak tissue produces the uredia or telia. About 7 to 10 days after inoculation, the uredia appear on the under surface of the oak leaf. The urediospore is the repeating stage of the fungus and urediospores are capable of reinfecting oaks but not pines. Telia appear 7-10 days after aeciospore inoculation. Telial columns may appear individually or in clusters, and thousands of telia may be found on one oak leaf. A telial column consists of independent teliospores bound together in a matrix. Teliospores are single-celled and thin-walled (Hedgcock and Siggers 1949), and may remain viable for several weeks. Under conditions of warm temperatures and high relative humidity (25± 2 °C and 97-100% RH), the teliospores germinate and produce a promycelium which bears four thin-walled, uninucleate basidiospores on short sterigma (Powers and Roncadori 1966). Nuclear fusion and meiosis occur prior to basidiospore formation. A highly variable population of abundant basidiospores is produced every year on oak (Snow et al. 1977), and different collections of this pathogen will vary widely in the ability to infect pines. Basidiospores are disseminated by wind to pines, penetrate susceptible pine tissues, and the life cycle begins again.

Ecology

Environmental factors, such as soil type, oak abundance, host phenology, susceptibility of varieties, pathogen fitness (either quantity or quality of pathotypes), and weather, strongly influence the incidence and severity of disease (Dinus and Schmidt 1977; Hollis and Schmidt 1977). Hollis and Schmidt (1977) found that fertile, well-drained soils where oak and pine grow best favored rust incidence. They suggested that the factor most associated with rust incidence was the amount of inoculum on the oak host.

Climatic conditions are a most important factor influencing rust incidence. Infections are favored by abundant periods of high relative humidity (≥ 97%), high leaf surface moisture either from rain or dew, and temperatures from 16 to 27 °C (Dinus and Schmidt 1977; Dwinell 1977; Snow and Froelich 1981).

Even though the amount and severity of infection depend on these interrelated phenomena, a major contributing factor to the increased incidence of fusiform rust is the increased growth of succulent pine tissues. Thus, additional site preparation and fertilization, for example, also can result in increased rust incidence (Schmidt et al. 1981).

Host Range

Forty-five species and cultivars of *Pinus* spp. were tested for susceptibility to fusiform rust. Of these, 26 of previous unknown susceptibility were susceptible to *C. quercuum* f. sp. *fusiforme*, and the susceptibility of 18 other species known to be susceptible was confirmed (Tainter and Anderson 1993). The most important species of southern pine attacked by *C. quercuum* f. sp. *fusiforme* are loblolly (*P. taeda* L.) and slash pines (*P. elliotii* var. *elliotii* Engelm).

Common natural primary hosts of *C. quercuum* f. sp. *fusiforme* include species of *Castanopsis, Lithocarpus, Castanea* and *Quercus*, with the genus *Quercus* being the most important (Czabator 1971; Dwinell 1977; Siggers 1955). Not all oaks are susceptible to the pathogen. Highly susceptible oaks include the black oak group: water oak (*Q. nigra* L.) and willow oak (*Q. phellos* L.) (Siggers 1955). Northern red oak (*Q. rubra* L.), southern red oak (*Q. falcada* var. *falcada* Michx), and blackjack oak (*Q. marilandica* Muench) are also important hosts to *C. quercuum* f. sp. *fusiforme*. In North Florida, water oak is the most abundant and susceptible oak species (Hollis and Schmidt 1977).

Geographic Distribution

Fusiform rust is distributed widely from southern Maryland to Florida and westward to Texas and southeast Arkansas (Phelps 1977).

The greatest disease incidence is found in the region extending from southeastern Louisiana through southern and central Mississippi, Alabama, Georgia, and central portions of South Carolina (Phelps 1977). In this rust corridor, disease incidence is higher in areas with intensive forest management (Schmidt et al. 1981), with up to 80% infection occurring in 8- to 12-year-old plantations of slash and loblolly pines.

Although the disease is not reported from other countries, species of *Cronartium* with oaks as alternate hosts are found in Canada, Mexico, Honduras and Guatemala (Czabator 1971).

Economic Importance

The disease significantly affects growth, causing deformities of the stem, and thus, reduces the timber market value, increases management costs, and can results in complete loss of plantations (Holley and Veal 1977). About one million acres of forest land are planted annually with slash and loblolly pines in the southeastern United States, and annual losses to the disease in these plantations are estimated to exceed $100 million (Schmidt et al. 1981). According to Webb and Patterson (1983), the average loss of utilizable sawtimber volume is 24-43% for loblolly pine and 23-53% for slash pine. The greatest loss, however, is attributed to the early infection and subsequent mortality or lack of growth (Holley and Veal 1977).

Control

Fusiform rust control strategies are based on an integrated pest management program (IPM). Many silvicultural options are available to forest land managers for reducing rust losses, including the evaluation of potential rust hazard at planting sites (which is assessed by the presence or absence of susceptible oaks) the amount of rust in nearby susceptible pine stands, the soil type, site intensity of site preparation, selection of planting stock, fertilization, and type of product that is to be produced (Schmidt et al. 1977; Schmidt et al. 1987; Schmidt et al. 1988; Schmidt et al. 1995).

In nurseries, seedlings can be protected from infection for 2 years after outplanting by application of the fungicide Triadimefon (Bayleton®) (Kelley et al. 1984). However, established galls do not respond to chemical treatment (Rowan 1982), and galled trees can not be recommended for reforestation or establishment of new plantations.

Although cultural practices that reduced planting density, selection of good planting stock, optimum fertilization, avoidance of planting rust-infected seedlings, and avoidance of high-hazard areas lowered the incidence of stem infection, reduced aeciospore production, and improved the quality of thinned stands (Belanger et al. 1991), the most economically feasible way to reduce fusiform rust incidence in plantations is to select and breed for resistance. Currently, reduced rust incidences may be achieved by planting rust-free resistant seedlings, and by avoiding the planting of highly susceptible slash and loblolly pines in areas of high rust incidence (Schmidt et al. 1981). Rust resistant seedlings can reduce rust incidences by 20 to 80% in these areas, with little or no reduction in growth (Pye et al. 1995).

In the 1960s, no rust resistant seedlings were available to forest land managers. Initially, the emphasis in tree improvement programs was on growth and yield traits; realized gains in these traits were 10-29% (Schmidt et al. 1981). Later, these gains and more were lost to fusiform rust. Rust was then recognized as a problem, and substantially more emphasis was placed on rust resistance tree improvement programs. For example, in slash pine the first generation orchards were genetically rogued in1988 (Hodge et al. 1989). The first evidence of differences in response to infection by fusiform rust on open pollinated progeny from a group of slash pines was demonstrated by Barber et al. (1957). Resistance was also found in hybrids from shortleaf and slash or loblolly pines (Henry and Bercaw 1956).

A screening program to find resistant varieties of slash pine was begun by the Cooperative Forest Genetics Research Program, at University of Florida, in 1969. The objectives of the program were to provide information on all selected families used in that breeding program through standard progeny tests and artificial inoculations. The initial selection was primarily based on growth parameters and form quality. Although the same progeny test allowed reliable identification of rust resistant families, not all tests were established in high-rust areas. Subsequently, progeny tests to reevaluate all initial selections for rust resistance were established in high-rust areas (Sohn et al. 1975). Resistance has been selected in both slash and loblolly pine species, and is been used operationally to control the disease in plantations (Schmidt et al.1985)

The first effective procedure for inoculating seedlings with the fusiform rust fungus was devised by Jewell (1957a). Inoculations were conducted using telia-bearing oak leaves distributed on a wire frame directly over the pine seedlings with water to maintain moisture. This work provided firm evidence that rust resistance existed in the selections of the tree improvement program. This method has been under constant improvement (Miller 1970; Powers et al. 1971; Snow and Kais 1972). A team of scientists at Athens, Georgia, for example, succeeded in developing an artificial inoculation system for mass-screening. It provides better control of inoculum concentration and standard conditions of host and environment (Matthews and Rowan 1972). The concentrated basidiospore spray technique (CBS) was established by the USDA Forest Service at Asheville, to screen large numbers of slash and loblolly pine seedlings for rust resistance (Anderson and Powers 1985). Generally, results from the CBS are in good agreement with field data; very resistant and very susceptible families of loblolly and slash pines are appropriately identified (Miller and Powers 1983).

Initially, percentage of galled seedlings was used at the Resistance Screening Center to evaluate resistance to fusiform rust. Fusiform rust resistance in pine was a measure of lower frequency of seedlings when compared with the frequency of seedlings with galls in susceptible (control) families (Kuhlman and Matthews 1993). Nevertheless, these galls may vary in size, from a slight swelling (short gall) up to two to three times the normal stem diameter (galls are large and fusiform shape). They also may vary in appearance; the gall surface is either smooth with the same texture as the unaffected stem tissue above or below the swelling, or the gall surface is rough, with reddish-brown necrotic and sunken areas (Miller et al. 1976; Powers 1971; Walkinshaw et al. 1980).

In the greenhouse, seedlings without stem swelling and with a purplish discoloration were observed for the first time on infected potted plants (Jewell 1957b). Whether or not these were symptoms of stem infections was not certain. Miller et al. (1976), studying the anatomy of inoculated slash pine seedlings, revealed four general types of host responses: (1) no penetration or infection, (2) subliminal infection, (3) three related types of hypersensitivity, and (4) typical gall development. The hypersensitive responses, which confined the fungus to reaction zones of necrotic tissue, were macroscopic and were correlated with the purplish lesions on the stem, which was considered to be an indication of resistance.

Walkinshaw et al. (1980) and Hubbard (1981), using data from the Resistance Screening Center, evaluated four forms of indices for use in predicting relative field resistance. They showed that the symptoms short gall, smooth gall, and rough gall could be used to predict relative levels of rust resistance of pine families in the field. Since then, gall shape and appearance have been used as further indications of resistance and susceptibility to fusiform rust. Currently, the Resistance Screening Center uses an index developed by Walkinshaw et al. (1980) that classifies levels of rust resistance. This method allowed the correlation of greenhouse traits to resistance in field tests; it also allowed the development of a selection index predictive of family rust resistance in field conditions.

Resistance to fusiform rust has been observed in natural and artificial inoculations of loblolly and slash pines, and their more resistant relatives (Goddard and Wells 1977). Significant variation in disease has been noticed among the rust collections from different states and among collections within individual states (Powers et al. 1977). Also, great variability was detected in a limited sample of the fungus population. Snow et al. (1976)

confirmed that this extensive variability is the product of past evolution, and that now foresters are dealing with genetic diversity that has evolved in natural populations of both the host and the pathogen.

In general, fusiform rust does not show great differences from other important plant disease fungi, such as cereal rusts. Since *C. quercuum* f. sp. *fusiforme* and its hosts co-evolved and were disturbed only recently, a substantial variety and number of resistant genes should be available (Dinus 1974). Improving genetic resistance for fusiform rust is complicated by variation in the fungus itself. However, a moderate degree of genetic control of fusiform-rust resistance has been found, with additive and nonadditive variation in slash and loblolly pines (Hodge et al. 1990; Kinloch and Stonecypher 1969). Also, genetic gains in rust resistance have been obtained. The nature of genetic variability of populations of the pathogen has been the subject of several molecular genetic studies, which indicated pathotype specific (vertical resistance). Indeed, strong evidence for vertical resistance was presented by Wilcox et al. (1996) with the identification of a major disease resistance gene in a loblolly pine family.

The use of resistant seedlings, the silvicultural options now available, and the wise deployment of resistant genes will help in reducing losses caused by damage to fusiform rust.

CHAPTER 2
TEMPORAL VARIATION IN FUSIFORM RUST INCIDENCE ON SELECTED RUST-RESISTANT FAMILIES OF SLASH PINE AND ISOLATES OF CRONARTIUM QUERCUUM F. SP. FUSIFORME

Introduction

Fusiform rust, caused by the fungus *Cronartium quercuum* (Miyabe ex Shirai) f. sp. *fusiforme*, is the most important disease of slash pine (*Pinus elliottii* var. *elliottii* Engelm.) and loblolly pine (*P. taeda* L.). Reduced rust incidences may be achieved by planting rust-free resistant seedlings (Schmidt et al. 1981). However, little is known about the nature of this resistance, especially the stability of rust-resistant pine families and distribution and frequency of virulent and avirulent pathotypes in the field.

In plantations, rust incidence on resistant slash pine families evaluated over time have been reported to be higher than expected, and rankings may sometimes be inconsistent (Rockwood and Goddard 1973; Schmidt and Allen 1991; Sohn et al. 1975; Sohn and Goddard 1979). The cause of this family by year interaction is still unclear. Also, little is known about pathogen stability over time. Most information is from survey data, which have suggested that yearly variation in fusiform rust incidence was due to weather conditions (Phelps 1977; Siggers 1949). Likewise, field experiments on the performance of slash pine families in high rust hazard areas show a significant year to year variation. Schmidt and Allen (1991) reported that rust incidence in slash pine plantations increased from 1961 to 1968, and decreased thereafter. Wave years occurred at 5-year intervals in 1963, 1968, 1972, and 1978; therefore, it was concluded that much of this variability was associated with year of planting. Similarly, Schmidt et al. (1985), studying 1,882 slash pine plantations established from 1961 to 1980, determined that, in areas of high rust potential, incidence generally remained high with each successive year of planting. However, both years and individual plantations with relatively low rust incidence occurred. It is difficult to draw conclusions about the temporal stability of resistant slash pine families and isolates of *C. quercuum* f. sp. *fusiforme* because many of these experiments: (1) were not repeated over time; 2) contained a limited number of families; or 3) did not use specific isolates of *C. quercuum* f. sp. *fusiforme*. Also there are several confounded elements in field studies that could be assigned to the cause of the increased rust incidence in resistant families.

Rust incidence in the field is closely related to climatic, edaphic, and biotic factors (Schmidt et al. 1981; Snow et al. 1977). Pine infection depends on the succulence and susceptibility of the new pine growth, when spores are present and environmental conditions are favorable for sporulation and infection. Site preparation and fertilization also can result in increased rust incidence (Schmidt et al. 1981). Infections of fusiform rust are favored by an abundant period of high relative humidity (≥ 97%), high leaf surface moisture (rain or dew), and temperatures from 16 to 27 °C (Dwinell 1977; Snow and Froelich 1981). These conditions are often favorable in most years in the high rust hazard areas and do not frequently limit rust development in these areas (Schmidt et al. 1981).

Pathogen variability is another factor that may contribute to increased rust incidence. Evidence of great genetic diversity was demonstrated in both the fungus and the host population (Snow et al. 1976). *Cronartium quercuum* f. sp. *fusiforme* is macrocyclic and heteroecious, alternating between pine and oak hosts (Czabator 1971; Dwinell 1977). Each spring its life cycle is renewed when aeciospores on pine are wind-disseminated to emerging leaves of the alternate oak host. Basidiospores produced on oak leaves can infect succulent pine tissues of the current year's growth; consequently, a highly variable population of basidiospores may be produced every year on oak leaves. It is expected that different populations of this pathogen will vary widely in the ability to infect pines (Powers et al. 1977; Snow et al. 1977). Variation in susceptibility among oaks occurs; northern red oak, as an example, supports more telia per leaf and more virulent basidiospores than water oak (Dwinell 1985). In areas of high rust incidences, rust may increase with each successive year of planting if selection for virulence occurs. Despite years with high or low rust incidences, rust incidence in slash pine plantations increased 2-3% in each successional year of planting from 1950 to 1965 (Schmidt et al. 1974) and from 1962 to1971 (Griggs and Schmidt 1977). In contrast, the reduced resistance, or increase of pathogen virulence, of a slash pine family was also reported (Snow et al.1976).

In 1987, "Rust Virulence" field trials to characterize the spatial and temporal variability in *C. quercuum* f. sp. *fusiforme* were established in the Coastal Plain of the southeastern United States. Progenies from open- and control-pollinated, rust-resistant and susceptible slash and loblolly pine families were planted in numerous locations of the coastal plain in 1987, 1989, 1991 and 1993 (Schmidt and Allen 1990). Each planting year was a completely randomized design with five replications (blocks) of 10-tree-row plots per family. Analyses of the cumulative rust incidence at 5 years of age in the slash pine plantations established in 1987, 89, and 91 showed a statistically significant resistant family by year interaction (Schmidt et al. 1999). This interaction was the result of a significant difference in rust incidences between families and years, with rust incidence increasing significantly from 1987 to 1989 in some families, but decreasing from 1987 to 1989 in other families, at the same locations. In the Rust Virulence plantings, where families were repeated with each planting, average rust among all families increased significantly from 15.1 to 21.2 % from 1987 to 1991 (Schmidt et al. 1999).

In plantations, rust incidence on selected resistant families has increased as rust increased on the susceptible families (Schmidt et al. 1995). Abundant evidence suggests that the observed differential response of pine progenies to rust with time and environment is a consequence of the extensive pathogenic variability observed in natural populations of *C. quercuum* f. sp. *fusiforme* (Dwinell 1985; Powers 1980; Snow and Kais 1970; Snow et al. 1975). This host-pathogen interaction comprises one of the potential problems in a rust-resistance breeding program, and is one possible cause of "breakdown" of genetic resistance as a result of an increase in frequency of virulent types of the pathogen. It is important to find the causes of this interaction, because they will have important implications for breeding and deployment strategies, and for future research on resistance. The cause of this interaction cannot be conclusively assigned from the Rust Virulence field studies. Cumulative rust incidences in 5-year-old trees may have been affected by many uncontrolled factors, such as soil characters, oak abundance, weather, host phenology, or inoculum potential.

It is hypothesized that variation in rust incidences from different years might be caused by pathogen variability, rather than by climate or phenology. The objective of this research was to study the effect on rust incidence of pathogen isolates collected from two time periods (years) from four locations in the Rust Virulence field tests. Artificial inoculation in the greenhouse at the Resistance Screening Center in Asheville, NC, was employed to control the independent variables present in the field tests.

Material and Methods

Families

Seeds of six open-pollinated slash pine progenies, obtained from seed collected for the Rust Virulence field tests, were used in this study (Figure 2-1). Their maternal parents were selected in the coastal plain of Florida and Georgia, and seed orchards were located in the same general area. The parents were chosen from a population previously selected based on their breeding values for rust resistance (R50) and volume growth. An R50 expresses the expected percent of offspring from a given parent that will be infected with rust on a field site in which unimproved material expresses 50% disease. The qualitative ratings and breeding values for rust incidences (R50) and volume growth of the maternal parents and the general location of selection of these parents are given in Table 2-1. Based on these ratings performance, families R1 and R4 were noted as resistant, families R2 and R3 were very resistant, and families S1 and S2 were very susceptible. The performance of these families in four field plantings (13, 17,19, and 20) in the Rust Virulence study appears in Table 2-2a and 2-2b.

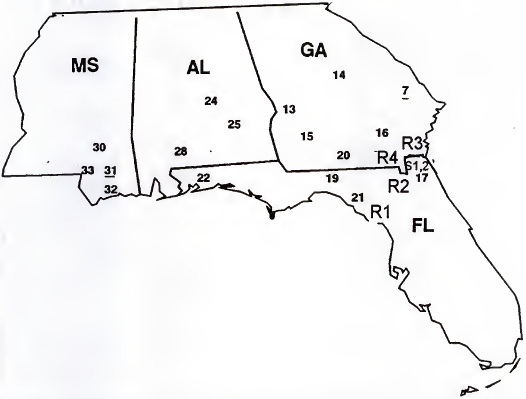

Figure 2-1.Seventeen locations of the rust virulent studies planted in the coastal plain of Florida, Georgia, Alabama, and Mississippi. Numbers in gray circles represent the locations (13, 17, 19, 20) selected to study the temporal and geographical variation in fusiform rust incidence among open pollinated slash-pine progenies. Locations from which the rust-resistant (R) and rust-susceptible (S) maternal parents were selected are shown as R1, R2, R3, R4, S1, and S2.

Table 2-1. Volume gains and fusiform rust incidence ratings (R50) of maternal slash pine parents, and the locations from where the seeds were collected.

Maternal Parents[v]	Locations[w]	BLP ratings		Qualitative ratings[z]	
		Vol. gain (%)[x]	R50[y]	Vol. gain (%)	Rust resistance
R1	Dixie Co., FL	14	20	high	resistant
R2	Columbia Co., Fl	23	4	very high	very resistant
R3	Charlton Co.,GA	2	5	average	very resistant
R4	Clinch Co.,GA	22	27	very high	resistant
S1	Nassau Co., FL	15	83	high	very susceptible
S2	Nassau Co., FL	13	82	high	very susceptible

[v] Represents resistant maternal parent, S represents susceptible parents.

[w] Locations from which the rust-resistant (R) and rust-susceptible (S) maternal parents were selected.

[x] Volume gain expressed as the percentage gain over unimproved material.

[y] R50 expresses the expected percent of offspring from a given parent that will be infected with rust on a field site in which unimproved material expresses 50% infection.

[z] Subjective ratings based on Best Linear Prediction (BLP) ratings.

Table 2-2a. Percentage of 5-year-old trees infected with fusiform rust; progenies included four resistant and one susceptible, open pollinated, slash pine families at four locations in the 1987 planting of the Rust Virulence field study.

	Families[y]					
Locations[x]	R1	R2	R3	R4	Mean	S2
13	41.1	18.7	25.1	30.7	38.9	69.1
17	10.7	10.0	12.0	6.0	9.67	60.3
19	27.3	12.9	50.8	34.0	31.25	90.0
20	29.0	18.4	36.6	27.6	27.90	97.5
Means[z]	27.0	15.0	31.1	24.6	24.43	97.5

[x] The numbers 13, 17, 19, 20 represent the locations selected to study the temporal and geographical variation in fusiform rust incidence among open pollinated slash-pine progenies.

[y] R1, R2, R3, and R4 represent resistant families; S2 represents a susceptible family.

[z] Means from five replicates of 10-tree-row plots.

Table 2-2b. Percentage of 5 year-old trees infected with fusiform rust; progenies included four resistant and one susceptible, open pollinated, slash pine families at four locations in the 1989 planting of the Rust Virulence field study.

	Families[y]					
Locations[x]	R1	R2	R3	R4	Mean	S2
13	51.1	35.5	-	-	43.6	-
17	14.9	8.40	24.9	14.2	15.6	74.4
19	28.5	24.7	24.6	17.1	23.7	96.6
20	7.3	9.7	19.00	13.2	12.30	93.1
Means[z]	27.0	19.6	22.8	14.8	20.7	87.8

[x] The numbers 13, 17, 19, 20 represent the locations selected to study the temporal and geographical variation in fusiform rust incidence among open pollinated slash-pine progenies.

[y] R1, R2, R3, and R4 represent resistant families; S2 represents a susceptible family

[z] Means from five replicates of 10-tree-row plots.

Isolates

Eight composite isolates were chosen representing four locations of the Rust Virulence experiment, 13, 17, 19, and 20 (location-isolates) (Figure 2-1), and two time periods, 1 and 2 (year-isolate), at each location. Each isolate was a composite of aeciospores collected from stem galls on trees in the two susceptible families: three collections from S1 and three from S2. Composite collections from the susceptible families were chosen since it was believed that these represented the most variable inoculum, representing the overall diversity of inoculum in the surrounding area. The locations were chosen based on (1) yearly variation in the Rust Virulence study for the 1987 and 1989 plantings, and (2) high rust-hazard areas, in order to have enough infected trees from which inoculum would be available. Collections representing the time periods were obtained by selecting stem galls from the two different planting years. For example, galls observed in 1998 on the lower stem from the 1987 planting represented older infections (inocula from 1989-1991); while galls from higher on the stem from the 1989 planting represented newer infections (inocula from 1992-1993). All aeciospore collections were processed separately and stored according to the procedures of Roncadori and Matthews (1966). In brief, aeciospores were collected from stem galls using a dual valve suction bulb; aeciospore collections were individually screened through a 50-mesh sieve to remove insects and trash; germination was checked; spores were desiccated and stored in ampoules with the aid of a vacumm pump tube; and ampoules were sealed with a natural gas-air torch, labeled and stored under refrigeration conditions. Prior to storage, each collection was checked for germination percentage, and assigned a number indicating date, location, and year of infection. The collections with the highest germination rate for each isolate were used to obtain the composite inocula. Inocula were stored and sent to the Resistance Screening Center, where the experiment was conducted.

Inoculation

To produce basidiospore inoculum, seedlings of northern red oak *(Quercus rubra* L.) were inoculated with aeciospores from each of the eight collections (four locations and 2 years). Later, basidiospores were harvested from oak leaves and used in a concentrated basidiospore spray system (CBS) for pine inoculations (Anderson and Mistretta 1982; Knighten et al.1988). Briefly, pine seeds were germinated, and the seedlings were transplanted immediately after emergence into flats containing 20 seedlings each. At 6 weeks of age, the seedlings were carried on a conveyor belt under a spray of an aqueous suspension of basidiospores. Inocula were adjusted to 20,000 spores per ml. Each of the eight rust collections were tested on 80 seedlings for each of the six families; thus 3,840 seedlings were inoculated, and, of these, 640 were checks of the Resistance Screening Center not included in the analysis (Table 2-3). After inoculation, seedlings were kept in an incubation chamber at 20 °C and 100% relative humidity, for 24 hours. Then, they were grown in the greenhouse for 6 months.

Artificial inoculations were conducted on 2 inoculation days as follow: on each day, families were randomly sprayed with inoculum of each basidiospore collection of *C. quercuum* f. sp. *fusiforme*. A mixture of rust-susceptible slash pine families from the Resistance Screening Center was used as a check for the inoculation efficiency.

Table 2-3. Slash pine families and *Cronartium quercuum* f. sp. *fusiforme* isolates employed to study temporal (year) variation in resistant slash pine families.

	Isolates[w]								
	13		17		19		20		
Family[v]	**Yr1**	**Yr2**	**Yr1**	**Yr2**	**Yr1**	**Yr2**	**Yr1**	**Yr2**	**Total**
R1	13-1[x]	13-2	17-1	17-2	19-1	19-2	20-1	20-2	640[y]
R2	13-1	13-2	17-1	17-2	19-1	19-2	20-1	20-2	640
R3	13-1	13-2	17-1	17-2	19-1	19-2	20-1	20-2	640
R4	13-1	13-2	17-1	17-2	19-1	19-2	20-1	20-2	640
S2	13-1	13-2	17-1	17-2	19-1	19-2	20-1	20-2	640
Total	400 [z]	400	400	400	400	400	400	400	3200

[v] R1, R2, R3, and R4 represent resistant families; S2 represents a susceptible family.

[w] Each isolate is a composite of three galls from S1 and S2.

Yr1 and Yr2 are isolates collected in different years: Yr1 are inocula from older galls infected in 1988-89, and Yr2 are inocula from younger galls infected in 1992-93.

The numbers 13, 17, 19, and 20 represent the locations of isolate collection selected to study the temporal and geographical variation in fusiform rust incidence among open pollinated slash-pine progenies.

[x] Cells indicate the specific isolates used (for example, 13-1= location 13, isolate collected from younger galls).

[y] 640= 2 years x 4 locations x 2 replications with 20 seedlings each x 2 inoculation days.

[z] 400 = 5 families x 2 replications with 20 seedlings each x 2 inoculation days.

Six months after inoculation, binary data (presence and absence) of several fusiform rust symptoms were evaluated. The traits were: (1) smooth gall (SO), the gall surface has the same texture as the unaffected stem tissue above and below the swelling; (2) rough gall (RO), the gall surface is rough and reddish-brown with obvious necrotic sunken areas; (3) large gall (LG), gall is more than 25 mm long; (4) small gall (SG), smaller or equal to 25 mm long; (5) purplish discoloration on the stem and no swelling (SYMNOS); (6) healthy (HE), seedlings with no apparent symptom, and (7) any gall type (GAL).

Experimental Design and Statistical Analyses

The factorial experiment was a split plot in a randomized complete block design with two replications. Whole plots were 2 year-isolates x 4 location-isolates. Families were subplot treatments (Table 2-3).

Analyses of variances were conducted for each group of isolates and symptom traits with and without the susceptible family S2, and although only the ANOVA without the susceptible family is presented (Table 2-4), multiple comparisons analysis were conducted considering the ANOVA with the susceptible family S2. ANOVA were conducted for isolate across locations and across and within families in a pooled analysis. Subsequently the effects of isolates was partitioned into location-isolates and year-isolates, and ANOVA were conducted for each group of isolates across and within families. As the interactions of inoculation day with family and inoculation day with family and isolates were not significant, they were pooled with the replication within the year x location x family interaction to generate the subplot error term in Table 2-4. Because the inoculation was conducted into two days, each with two replications, the interactions with days could not be pooled to estimated the whole plot error. The contributions of isolates and family to the family by isolate and family by location-isolate interaction sum square were calculated according to Mandel (1971). Inoculation days were considered as a random effect and all other factors as fixed. The binomial traits (SYMNOS, GAL, HE, SO, RO, and SG) were analyzed as plot means (percentages). The percentages of seedlings with small galls (PSG), rough galls (PRO), and smooth gall (PSO) were found by dividing the number of plants with these symptoms by the total number of plants with galls. The

Table 2-4. Full model used for analysis of variance (4 families x 2 replicates x 8 isolates x 2 inoculation days to assess temporal (year) variation in resistant slash pine families.

Source of variation	DF[r]	Expected Mean Squares
Isolates (I)[s]	7	$\sigma^2_e + 8\,\sigma^2_{DI} + \sum^{t}(I)$
Year-isolate (Yr)	(1)	$\sigma^2_e + 8\sigma^2_{YrDL} + 32\,\sigma^2_{ID} + \sum(Yr)$
Location-isolate (L)	(3)	$\sigma^2_e + 8\,\sigma^2_{YrDL} + 16\,\sigma^2_{DL} + \sum(L)$
Yr*L	(3)	$\sigma_e^2 + 8\,\sigma^2_{YrDL} + \sum(I*L)$
Day (D)[u]	1	$\sigma^2_e + 8\sigma^2_{DI} + 64\,\sigma^2_D$[v]
I * D	(7)	$\sigma^2_e + 8\sigma^2_{DI}$[w]
Yr*D	(1)	$\sigma^2_e + 8\,\sigma^2_{YrDL} + 32\,\sigma^2_{DI}$[w]
L*D	(3)	$\sigma^2_e + 8\,\sigma^2_{YrDL} + 16\,\sigma^2_{DL}$[x]
Yr* L*D	(3)	$\sigma^2_e + 8\,\sigma^2_{YrDL}$[y]
Family (F)	3	$\sigma^2_e + \sum(F)$
F*I	21	$\sigma^2_e + \sum(FI)$
F*Yr	(3)	$\sigma^2_e + \sum(F*Yr)$
F*L	(9)	$\sigma^2_e + \sum(F*L)$
F*Yr*L	(9)	$\sigma^2_e + \sum(F*Yr*L)$
Subplot error	88	σ^2_e[z]

[r] Degrees of freedom.

[s] Isolates are year- and location-isolates. Year-isolates are from time periods 1 (1988-89) and 2 (1992-1993); location-isolates are isolates from locations 13, 17, 19, and 20.

[t] $\sum$ represents fixed effect

[u] Days are inoculations conducted on each of 2 consecutive days.

[v] σ^2_D = estimated variance of inoculation day for a given variable.

[w] σ^2_{DI} = estimated variance of inoculation day x isolates x day interaction for a given variable.

[x] σ^2_{DL} = estimated variance of inoculation day x location-isolates interaction for a given variable.

[y] σ^2_{YrDL} = estimated variance of year- x location-isolates and inoculation day interaction for a given variable.

[z] σ^2_e = estimated variance of year x location x family interaction pooled with the interactions of inoculation day with family and inoculation day with family and isolates (subplot error).

percentages of seedlings with large gall (PGAL) and SYMNOS (PSY), and of healthy plants (PHE), were obtained by dividing the number of plants with these symptoms by the total number of plants. All traits were analyzed as untransformed plot means of the percentage of infected resistant trees, since arcsin transformation did not provide any improvement in the results.

Only the susceptible variable PGAL, and the resistant variables PRO, PSG, and PSY are presented. The results for PHE and PSO are not presented because the former is nearly the inverse of PGAL, and the second is the inverse of PRO. The results are presented as main effects, and as first order interactions with main effects.

To investigate the pattern of variation for isolate, the sum of squares for isolates was partitioned into effects of year-isolates and location-isolates, with 1 and 3 degrees of freedom; respectively. Duncan's multiple range test determined if significant differences existed between year-isolates within families and within year-isolate among families. All statistical analyses were conducted with SAS® (SAS Institute Inc. 1989).

Results

Overall Means

The overall test mean for percentage of seedlings with any type of gall (PGAL) was high for both susceptible checks; 92% for the Resistance Screening Center check and 95% for the rust-susceptible S2, indicating successful inoculations.

Family Effect

The differences among slash pine families were highly significant (p=0.0001) for all traits of gall development (Table 2-5). Resistant families R1 and R3 had higher mean values for the resistant variables PSY, PRO, and generally for PSG, and lower means for PGAL, than the resistant family R4 and the susceptible family S2 (Table 2-6).

No statistical differences in PGAL, PSY, and PRO were found among the resistant families R4 and R2, and the susceptible family S2, indicating that families R4 and R2 were not as resistant as the other resistant families (Table 2-6). However, significant differences were found between families R3 and R1, for PGAL and PSG. For example, based on PGAL, R3 was the most resistant family (54% with galls) followed by R1 (75% with galls). Although no statistical differences were found between S2 and R2 for PGAL, family R2 had the highest percentage of seedlings with short galls (54%). This may explain the ranks obtained with the field prediction index for rust resistance, in which family R2 generally ranked either first or second at most locations (Table 2-7). This result also agrees with the field data, where R2 ranked first or second at most locations and plantations (Table 2-2a, b).

Year-Isolate Effect

Overall, no significant effect of year-isolates was found (Table 2-5). For example, for PGAL, year-isolate averages (across locations) were remarkably similar (Table 2-8): 79% for isolate Yr1 and 81% for isolate Yr2. This trend was consistent across all variables. Nevertheless, within families R3 and R4 a significant difference in disease

Table 2-5. Mean square and significance probability values (within parenthesis) for split plot analysis of variance describing temporal (year) variation in fusiform rust incidences after artificial inoculations with isolates of *Cronartium quercuum* f. sp. *fusiforme* at the Resistance Screening Center on open-pollinated progeny of rust-resistant slash pine families.

Sources of variation	**DF**[w]	**PGAL**[x]	**PSY**[x]	**PRO**[x]	**PSG**
Isolates (I)[y]	7	375.85	116.86	127.12	50.34
		(0.24)	(0.57)	(0.12)	(0.97)
Year-isolate (Yr)	(1)	246.44	63.41	53.21	53.02
		(0.45)	(0.47)	(0.17)	(0.77)
Location-isolate (L)	(3)	347.45	194.5	205.25	21.07
		(0.49)	(0.57)	(0.03)	(0.33)
Yr*L	(3)	447.39	57.05	73.63	78.73
		(0.13)	(0.49)	(0.59)	(0.89)
Day (D)[z]	(1)	180.46	85.6	310.5	169398
		(0.57)	(0.60)	(0.10)	(0.99)
I * D	(7)	215.80	134.61	50.11	228.16
		(0.02)[v]	**(0.03)**	(0.96)	(0.16)
Yr*D	(1)	176.33	51.58	4.17	386.92
		(0.28)	(0.41)	(0.85)	(0.39)
L*D	(3)	341.85	240.64	15.85	12.06
		(0.17)	(0.13)	(0.92)	(0.99)
Yr.* L*D	(3)	102.91	56.27	99.68	391.33
		(0.32)	(0.41)	(0.64)	(0.52)
Family (F)	3	8733.78	417.90	3584.39	10431.9
		(0.0001)	**(0.0001)**	**(0.0001)**	**(0.0001)**
F*I	21	248.47	73.69	230.75	202.34
		(0.0003)	(0.21)	(0.19)	(0.15)
F*Yr	(3)	119.23	227.84	308.82	454.23
		(0.0001)	**(0.01)**	(0.16)	(0.03)
F*L*	(9)	84.90	47.10	331.68	195.77
		(0.46)	(0.60)	(0.06)	(0.23)
F*Yr*L	(9)	121.79	48.91	103.79	124.93
		(0.19)	(0.57)	(0.8)	(0.57)
Subplot error	88				

[v] Numbers in bold are significant at the 5% level.

[w] Degrees of freedom.

Table 2-5--continued

[x] PGAL, PSY, PRO, PSG are the variables used to measure phenotypic responses of slash pine families to *Cronartium quercuum* f. sp. *fusiforme*:

PGAL = percentage of seedlings with any gall type;
PSY = percentage of seedlings showing area of purplish discoloration and no stem swelling;
PRO = percentages of PGAL seedlings with a gall having an area of discoloration covering more than 50 % of the gall making it rough; and
PSG = percentages of PGAL seedlings with galls smaller than or equal to 25 mm long.

[y] Isolates are year- and location-isolates. Year-isolates are from time periods 1 (1988-89) and 2 (1992-1993); location-isolates are isolates from locations 13, 17, 19, and 20 in Florida and Georgia.

[z] Days are inoculations conducted on each of 2 consecutive days.

Table 2-6. Overall mean percentages for progeny of open-pollinated slash pine families with four phenotypic responses after artificial inoculation with *Cronartium quercuum* f. sp. *fusiforme*.

	Phenotypic responses [y]			
Families[x]	**PGAL**	**PSY**	**PRO**	**PSG**
R1	75 b [z]	15 a	77 b	48 b
R2	88 a	8 b	90 a	54 a
R3	54 c	13 a	72 b	18 c
R4	89 a	8 b	93 a	23 c
S2	94 a	3 b	88 a	6 d

[x] R1, R2, R3, R4 represent resistant families; S2 represents a susceptible family.

[y] Phenotypic responses of slash pine families to *Cronartium quercuum* f. sp. *fusiforme*:

PGAL = percentage of seedlings with any gall type type;
PSY = percentage of seedlings showing area of purplish discoloration and no stem swelling;
PRO = percentages of PGAL seedlings with a gall having a of discoloration covering more than 50 % of the gall making it rough; and
PSG = percentages of PGAL seedlings with galls smaller than or equal to 25 mm.

[z] Values within column followed by the same lowercase letter do not differ statistically according to Duncan's multiple range test (P=0.05).

Table 2-7. Comparisons of fusiform rust incidence variables for greenhouse inoculations and field performance of progeny of slash pine families.

		Greenhouse				Field data					
		Index[u]		PGAL[v]		1987 Plantation [w]		1989 Plantation		1991 Plantation	
Location[s]	Fam[t]	Yr1[x]	Yr2	Yr1	Yr2	Incidence [y] (%)	R/S [z]	Incidence (%)	R/S	Incidence (%)	R/S
13	R1	135.1(2)	152.9(1)	73.6	71.3	41.1(4)	0.53(4)	51.7(2)	0.56(2)	26.2(3)	0.31(3)
13	R2	119.6(3)	131.6(3)	91.1	87.4	18.8(1)	0.24(1)	35.3(1)	0.39(1)	25.8(2)	0.30(2)
13	R3	174.6(1)	151.5(2)	39.3	60.3	25.1(2)	0.33(2)	-	-	-	-
13	R4	72.4(4)	131.1(4)	92.5	83.8	30.7(3)	0.39(3)	-	-	6.1(1)	0.07(1)
13	S2	63.6(5)	89.3 (5)	95.0	93.8	69.1(5)	-	-	-	78.6(4)	-
17	R1	103.6(4)	177.1(2)	83.8	67.0	10.7(2)	0.17(3)	14.9(3)	0.20(3)	26.1(2)	0.33(3)
17	R2	153.1(2)	177.3(1)	86.1	78.4	10.0(2)	0.16(2)	8.4(1)	0.11(1)	24.5(1)	0.30(2)
17	R3	153.8(1)	136.6(4)	41.4	49.9	12.0(4)	0.19(4)	24.9(4)	0.33(4)	-	-
17	R4	114.1(3)	147.7(3)	92.5	82.5	6.0(1)	0.09(1)	14.2(2)	0.19(2)	30.2(3)	0.37(1)
17	S2	48.9(5)	58.43(5)	93.8	97.5	60.3(5)	-	74.4(5)	-	86.7(4)	-
19	R1	127.8(1)	150.1(2)	72.0	78.6	27.3(2)	0.30(2)	28.5(4)	0.31(3)	50.2(3)	0.53(2)
19	R2	129.6(2)	159.5(1)	91.0	90.0	12.9(1)	0.14(1)	24.7(3)	0.27(2)	22.4(1)	0.53(2)
19	R3	90.0(3)	111.7(4)	52.7	66.3	50.8(4)	0.56(4)	24.6(2)	0.27(2)	52.2(4)	0.55(3)
19	R4	76.8(4)	145.6(3)	88.5	87.5	34.0(3)	0.38(3)	17.1(1)	0.19(1)	29.2(2)	0.31(1)
19	S2	46.8(5)	35.1(5)	91.2	97.4	90.0 (5)	-	96.0(5)	-	96.9(5)	-
20	R1	144.5(3)	163.1(1)	68.7	84.9	29.0(3)	0.31(3)	7.3(1)	0.08(1)	28.1(2)	0.30(2)
20	R2	148.2(1)	133.1(3)	87.2	96.1	18.4(1)	0.20(1)	9.7(2)	0.15(3)	29.6(3)	0.32(3)
20	R3	146.6(2)	106.3(4)	42.4	77.2	36.6(4)	0.39(4)	19.0(4)	0.22(4)	-	-
20	R4	89.4(4)	146.8(2)	98.8	85.0	27.6(2)	0.30(2)	13.2(3)	0.11(2)	22.0(1)	0.24(1)
20	S2	57.3(5)	49.6(5)	96.0	91.3	97.5(5)	-	93.1(5)	-	91.9(4)	-

[s] 13, 17, 19, and 20 are location-isolate collections from locations in Florida and Georgia.

[t] R1, R2, R3, R4 are resistant families; S2 is a susceptible family.

Table 2-7--continued

[u] Index (I) represents a value used to predict field performance of slash pine families to fusiform rust resistance.

$I = 25[PSY - M_1/S_1 + PRO - M_2/S_2 + PSG - M_3/S_3 + PHE - M_4/S_4]$, where

S is the standard deviation for each symptom;

M is the minimum value for each symptom over treatments;

PSY is the percentage of seedlings showing area of purplish discoloration and no stem swelling;

PRO is the percentages of PGAL seedlings with a gall having an area of discoloration covering more than 50 % of the gall making it rough; and

PSG is the percentages of PGAL seedlings with galls smaller or equal to 25 mm long.

[v] PGAL is the percentage of seedlings with any gall type.

[w] Plantation represents the year which a plantation was first established.

[x] Yr are year-isolates, Yr1 and Yr2 represent a composite collections (year-isolate) of three galls from S1 and S2. Yr1 is from older galls, 1988-89. Yr2 is from younger galls, 1992-93.

[y] Percent of trees infected with fusiform rust at age 5 yr.

[z] R/S represents the ratio of proportion of trees infected with fusiform rust on resistant (R), open-pollinated, slash pine families to that of trees infected on two susceptible (S), open-pollinated slash pine families at age 5 years.

Table 2-8. Phenotypic responses to *Cronartium quercuum* f. sp. *fusiforme* on five progeny of open pollinated slash pine families 6 months after artificial inoculation

	Phenotypic Responses [v] (%)							
	PGAL		PSY		PRO		PSG	
Families [w]	**Yr[x]1**	**Yr2**	**Yr1**	**Yr2**	**Yr1**	**Yr2**	**Yr1**	**Yr2**
R1	74 Ab[y]	75 Ac	15 Aa	16 Aa	75 Ab	79 Ab	48 Ab	49 Aa
R2	89 Aa	88 Aab	7 Aa	9 Ab	88 Aa	93 Aa	59 Aa	50 Ba
R3	44 Bc	63 Ad	15 Aa	10 Ab	77 Ab	67 Bc	15 Ac	21 Ab
R4	93 Aa	85 Bb	4 Bb	13 Ab	93 Aa	92 Aa	19 Ac	27 Ab
S2	94 Aa	95 Aa	3 Aa	3 Ab	87 Aa	89 Aa	7 Ad	6 Ac
mean[z]	79 A	81 A	9 A	10 A	84 A	84 A	30 A	30 A

[v] Phenotypic responses of slash pine families to *Cronartium quercuum* f. sp. *fusiforme*:
PGAL is the percentage of seedlings with any gall type;
PSY is the percentage of seedlings showing area of purplish discoloration and no stem swelling;
PRO is the percentages of PGAL seedlings with a gall having an area of discoloration covering more than 50 % of the gall making it rough; and
PSG is the percentages of PGAL seedlings with galls smaller or equal to 25 mm long.

[w] R1, R2, R3, and R4 represent resistant families; S2 represents a susceptible family.

[x] Yr1 and Yr2 are isolates collected in different years: Yr1 are inocula from older galls infected in 1988-89, and Yr2 are inocula from younger galls infected in 1992-93.

[y] Values within column followed by the same lowercase letter and within a row followed by the same uppercase letter do not differ significantly according to Duncan's multiple range test (P=0.05).

[z] Each value is the mean of two replicates and 2 inoculation days within a year-isolate across all families and locations (1600 trees).

incidence was observed between inoculum from different time periods. For example, within family R4 inoculum from isolate Yr2 were more virulent than inoculum from isolate Yr1 (Table 2-8). The opposite trend was observed within family R4, inoculum from year 1 was more virulent than inoculum from Yr 2.

Family by Year-Isolate Effect

A significant family by year isolate interaction occurred for PGAL (p=0.0001), PSG (p=0.03) and PSY (p=0.01) (Table 2-5). This interaction indicated that these families did not react the same when inoculated with isolates collected at different time periods.

Additional analyses showed that most of this family by year interaction was attributed to R3, which contributed 46-69% of the variation for all variables, except for PSG (21%) (Table 2-9A and 2-9B).

Differences in virulence between year-isolates are shown Table 2-8. Significant differences between inoculum sources were found within R3 for PGAL and PRO, with isolate from Yr2 being more virulent than isolate from Yr1 (Table 2-8). The opposite response was observed within R4 for PGAL, for which inoculum from Yr1 was more virulent than inoculum from Yr2. For R2 a significant difference between year-isolates was observed for PSG, with inoculum from Yr1 inducing more short galls than inoculum from Yr2.

Differences among families within inoculum sources were also observed (Table 2-8). Rust incidences were higher on R4, R2, and S2 than on R1 and R3 for PGAL and PRO. Families R1 and R2 had the highest rust incidences for PSG. Probably, most of the variation contributing to this interaction was from inoculum from Yr2, as more variability between Yr1 and Yr2 was detected on the resistant R3 and R4 families than on the other resistant families.

The differential response of families R3 and R4 with inocula from different time periods (Table 2-8) can be better visualized in Figure 2-2A, where, for PGAL, inoculum from Yr1 was more virulent on R4 than was inoculum from isolate Yr2, whereas on family R3 inoculum from Yr1 was less virulent than the inoculum from Yr2. Opposite reactions were observed for PSY, since inoculum from Yr1 was significantly more virulent on R3 than inoculum from Yr2, whereas inoculum from Yr1 was less virulent than inoculum from Yr2 on R4 (Figure 2-2B). The differences between inoculum sources within families emphasize the variability among isolates from different time periods.

Table 2-9. Percentage contribution of open pollinated slash pine progenies to interactions of family with year- and location-isolate, and family with location-isolate 6 months after artificial inoculation with *Cronartium quercuum* f. sp. *fusiforme*. Values in bold represent the highest family contribution to the interaction.

		Percentage of variation[z]			
Source of variation	**Family [y]**	**PGAL**	**PSY**	**PRO**	**PSG**
A) Family by isolate interaction (year- and location-isolate)					
	R1	9.3	6.5	9.2	29.2
	R2	32.4	41.7	8.9	18.2
	R3	**54.0**	**46.1**	**64.2**	21.0
	R4	4.3	5.8	17.8	**31.5**
B) Family by location-isolate interaction					
	R1	25.3	9.0	5.6	9.0
	R2	**14.8**	**20.7**	**13.6**	20.7
	R3	**47.8**	**52.4**	**69.5**	52.4
	R4	12.1	17.9	11.3	17.9

[y] R1, R2, R3, and R4 represent resistant families; S2 represents a susceptible family.

[z] Percentage contribution of slash pine families to *Cronartium quercuum* f. sp. *fusiforme*:
PGAL is the percentage of seedlings with any gall type;
PSY is the percentage of seedlings showing area of purplish discoloration and no stem swelling;
PRO is the percentages of PGAL seedlings with a gall having an area of discoloration covering more than 50 % of the gall making it rough; and
PSG is the percentages of PGAL seedlings with galls smaller or equal to 25 mm long.

Family by Location-Isolate Effect

Generally, inocula within isolates were statistically less virulent on family R3 than on R2, R3 and R4 ($P<0.05$) (Figure 2-3A). Moreover, substantial variability of the response of R3 was observed over locations, and within location between years. For example, within location 13 and 20, inoculum from isolate Yr2 was often more virulent on

R3 than inocula from isolate Yr1. No significant differences between year-isolate were found among the other locations. Despite this variable response of R3 to these inocula, R2, R1, and R4 responded equally to all isolates. The differential response of R3 to year and location-isolates is an indication of the differential pathogenicity of *C. quercuum* f. sp. *fusiforme* among slash pine families. Although no significant differences among locations and within year-isolate occurred, more variability in rust incidence among locations seemed to have occurred with inoculum from Yr2 than with inoculum from Yr1 (Figure 2-3B).

A correlation matrix for all symptoms is presented in Table 2-10. A negative correlation between PGAL and PSY was observed (r=-0.68), indicating an inverse relationship between susceptible (GAL) and resistant (SY) symptoms. Linear regression between PGAL and PSY for each family reinforces this result (Figure 2-4); the resulting regressions and the slopes were significant for all families. Slope coefficients were very similar (≈-1.0) for resistant and susceptible families. The coefficients of determination increased with susceptibility, indicating that for families with higher frequencies of galls, the association between these two traits tends to be more predictable. For example, the resistant R2, with y_{max}= 97 %, had $R^2 = 0.78$, while the most resistant R3, with y_{max}= 68.8%, had R^2=0.54. These results agree with the suggestion of Walkinshaw and Anderson (1983) that SYMNOS and small galls are indicative of resistance, whereas large galls are indicative of susceptibility.

Table 2-10. Estimation of Pearson correlation coefficients, based on 160 plot means, on progeny of open-pollinated slash pine for all possible pairs of the variables evaluated 6 months after artificial inoculations with *Cronartium quercuum* f. sp. *fusiforme*.

	Pearson Correlation Coefficient			
	PGAL	**PSY**	**PRO**	**PSG**
PGAL[w]	1.00	-0.68***	-0.89 ***	0.023 [ns]
PSY[x]		1.00	-0.20 ***	-0.20 ***
PRO[y]			1.00	-0.023[ns]
PSG[z]				1.00

*** The correlation is significantly different from zero at 0.0001 level.

[ns] The correlation is nonsignificant.

[w] PGAL is the percentage of seedlings with any gall type type.

[x] PSY is the percentage of seedlings showing area of purplish discoloration and no stem swelling.

[y] PRO is the percentages of PGAL seedlings with a gall having a of discoloration covering more than 50 % of the gall making it rough.

[z] PSG is the percentages of PGAL seedlings with galls smaller or equal to 25 mm.

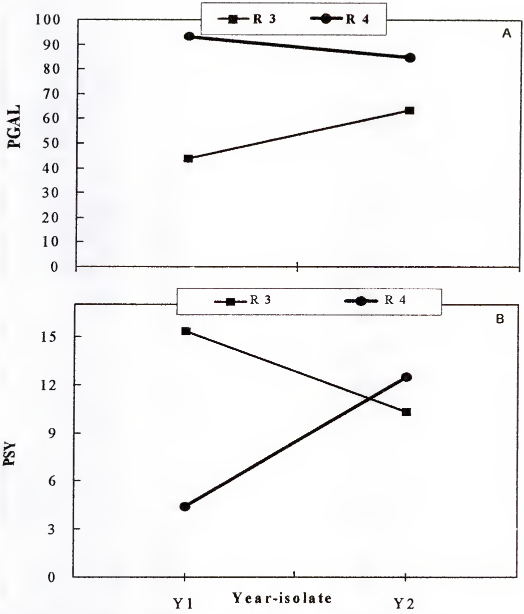

Figure 2-2. Family (R3 and R4) by year-isolate (Yr1 and Yr2) interaction (A) of percentage of seedlings with galls (PGAL) and (B) of percentage of seedlings with symptom and no swelling (PSY) on progeny for open-pollinated slash pine families inoculated with *Cronartium quercuum* f. sp. *fusiforme.* R3, and R4 represent resistant families; Year-isolates are from time periods 1 (1988-89) and 2 (1992-1993).

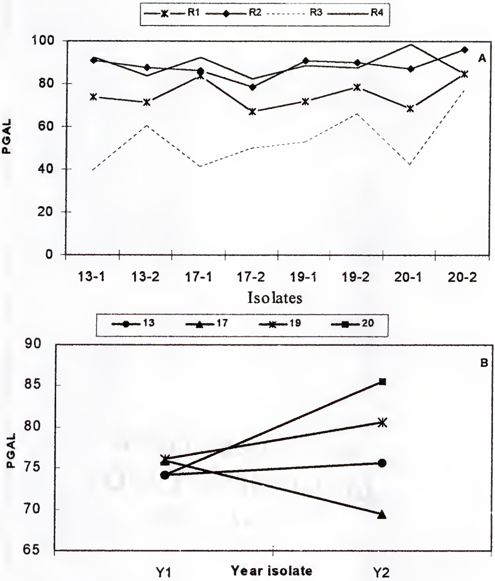

Figure 2-3. Family by isolate interaction (A) and family by location- (13, 17, 19, and 20) by year- isolate (Yr1 and Yr2) interaction (B) on percentage of seedlings with galls (PGAL) on progeny of open-pollinated slash pine families inoculated with *Cronartium quercuum* f. sp. *fusiforme*. R1, R2, R3, and R4 represent resistant families; S2 represents a susceptible family. Isolates are year- and location-isolates. Location-isolates are collections from locations 13, 17, 19, and 20 in Florida and Georgia, Yr1 and Yr2 are isolates collected in different years: Yr1 are inocula from older galls infected in 1988-89, and Yr2 are inocula from younger galls infected in 1992-93. Within an isolate, family R3 was statistically different from families R1, R2, and R4 ($P<0.05$).

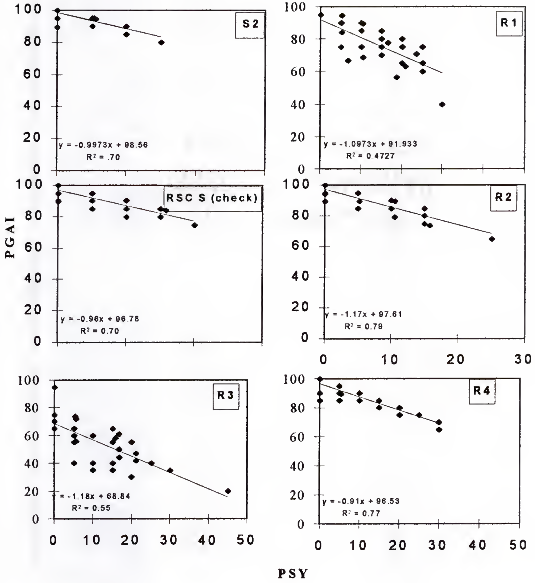

Figure 2-4. Relationship between percentage of trees with galls (PGAL) and percentage of trees with purplish discoloration on the stem and no swelling (PSY) on susceptible (S) and resistant (R) open pollinated slash pine families evaluated 6 months after inoculation with *Cronartium quercuum* f. sp. *fusiforme*. Each point represents the mean of 32 observations (2 year-isolates, 4 location-isolates, 2 inoculation days and replications).

Discussion

Artificial Inoculations

Although artificial inoculation in the greenhouse was employed to control the potential causal factors of the family x year interaction present in the field trials, variability is still present in the greenhouse data. The inocula used herein (six galls: three each from the two susceptible families) may approach the wild type; thus, it should represent the most variable inoculum in the surrounding area. Another source of variability is the open-pollinated slash pine families. The heterozygosity in open-pollinated families and the diversity within fungal isolates could account for some of the variability in the data, and probably contributed to the results. The problem of limiting heterogeneity in the host and in *C. quercuum* f. sp. *fusiforme* has long been challenging, and can best be accomplished with single spore fungal cultures and clonal host material.

The high disease level in some of the resistant families might be the result of: 1) increased disease with increased amounts of inoculum (for example, it could be that the inoculum density used in this study was too high, as some resistant families did not separate from the susceptible family); 2) a host response to increased selection pressure, leading to a gradual "erosion" of resistance, as previously reported by Sohn & Goddard (1979); 3) random recombination in the pathogen that may have led to selection of more virulent isolates; or 4) the favorable timing of biological events, such as phenology (very susceptible tissue) and basidiospore availability, which are conducive to greater disease under greenhouse conditions than in field trials. Although all of these factors may result in decreased resistance of relatively resistant families, this result emphasizes the importance of planting more than one family to control rust. The use of mixed families reduces the chances of a buildup or selection of virulent isolates, as previously suggested by Schmidt and Allen (1997). It also underscores the utility of the Resistance Screening Center index to evaluate field resistance.

The main objective of this research was to determine whether there was a temporal variation in the rust population of *C. quercuum* f. sp. *fusiforme* and to investigate the cause of the family x year interaction for progeny of resistant slash pine families. This question is important because it influences breeding strategies and deployment of resistant varieties. Duncan's comparisons between years isolates and within family indicated that there are temporal effects and that some families do react differentially to inoculum from different time periods (Yr1 vs Yr2), and, despite the variability in the data, pathogen variability appears to be a primary cause of this interaction (Table 2-8).

The significant family x year interaction for all traits is important. The interaction of rust incidences among families with inoculum from Yr1 and Yr2 indicates that the rust varies in virulence in different years, and that some families are not stable over time. Some families were quite stable in response; they proved susceptible to both inocula, whereas some families, R3 for example, were quite resistant to inoculum from Yr1 (44% disease) and moderately resistant to inoculum from Yr2 (63% disease). These results demonstrate variability between inocula from different years, and among families within inoculum from the same years. Overall, however, across all families and locations, years were not significant. This finding agrees with a previous study in loblolly pine, in which variability in rust incidence was less among old collections of *C. quercuum f. sp. fusiforme* and was not statistically significant (Powers and Dwinell 1978), it was suggested that these results likely were confounded by the variation in inocula and families. Also, the highest order interaction of family-by-year-isolates might have hidden the effect of year-isolate. In this study, analyses within families indicated that infection percentage produced with inocula from Yr1 and Yr2 differed among resistant families. For example, infection on R3 increased significantly with inocula from Yr2 compared to inocula from Yr1, indicating variability between the two rust populations. This finding is important because it could indicate that the younger rust population has a rapid ability to respond to changes in the host population, or it could result from random recombination in the pathogen. Moreover, additional analysis showed that significant disease variation between years was evident for some pine families, ranging from 44 to 94% disease, and that most of this variation was attributable to differences within R3 (Table 2-8). The resistance of families with such instability is likely to be overcome by extant virulent forms or by future shifts in virulence of the rust population. Other resistant families, such as R2, were remarkably uniform in their response to year-isolates over time.

These results support other researchers who found family and family x year effects in field or laboratory studies (Goddard and Schmidt 1979; Snow et al.1975; Snow and Kais 1970; Sohn et al. 1975); most have suggested that the primary cause of these effects was pathogenic variability. However, in field studies, the cause of family variation with year could not be confirmed (Goddard and Schmidt 1979; Schmidt and Allen 1997; Sohn and Goddard 1979). These authors have suggested that a year effect may have occurred because of differences in planting years, known as wave years, due to the effect of climate on inoculum production or phenology. In the present study, the fact that differences between the two inoculum sources were detected within some families is of primary importance because it suggests that *C. quercuum* f. sp. *fusiforme* may shift in virulence; it also provides strong evidence that pathogenic variability is the cause of the family x year interaction.

The significant variation in rust incidences among families was expected since families were previously selected for their different levels of resistance. However, family means across year- and location- isolates indicated that there were differences in rust incidence among resistant families. Percentage of seedlings with galls ranged from 54% to 94%, with R3 being consistently the most resistance for all variables, except PSG (Table 2-6). Typically, families separated into three groups: most resistance, R3; moderately resistance, R1; and susceptible, R2, R4 and S2. This segregation changed with symptom type, with R2 being the most resistant for PSG, but as susceptible as S2 for PGAL. The susceptibility of R2 was observed only when PGAL was considered; however, this family seems to be a good source of field resistance when the Resistance Screening Center index is considered. In fact, field evaluations have confirmed this response (Schmidt et al. 1999). Thus, the present study indicates the risk of testing slash pine families for resistance without careful regard to symptom type. For example, resistance of R2 might be underestimated if one only considers the typical response PGAL. Also, it indicates that patterns of symptom frequencies varied significantly among families, with the largest amount of variation occurring in PSG. The evaluation of families for relative frequencies of the four symptom types may indicate different response mechanisms among the resistant families. For PSG, for example, significant differences between year-isolate collections were found only in R3. As the same inoculum sources were used to compare these families, it seems probable that small galls are due to resistance in the host genotype and not due to variations in virulence in the pathogen. The frequency of SYMNOS varied the least (from 3 to 15%); with the highest and the lowest frequency of SYMNOS occurring in families with low and high frequencies of galls, respectively (Table 2-6). Conversely, comparable data concerning symptom expression among open-pollinated slash pine progenies after inoculation with *C. quercuum* f. sp. *fusiforme* (Kuhlman and Powers 1991) indicated that both the highest and the lowest frequency of SYMNOS occurred in a family with low frequency of galls. The variation in the frequency of SYMNOS was attributed to the heterozygosity in the open-pollinated material. Nevertheless, additional analyses indicated a negative relationship between PGAL and PSY (Table 2-10). Further, regression analysis indicated that increasing gall frequency and decreasing SYMNOS frequency reflect decreasing resistance (Figure 2-3). Thus, the observed variation between the two groups of families indicates that the frequency of SYMNOS is indeed a resistant reaction as previously suggested by Miller et al. (1976).

Comparisons with Rust Virulence Field Data

The results of the Rust Virulence study support the results of this study, where family x year interaction was statistically significant. Briefly, the Rust Virulence study showed that (1) rust increased in Yr3-Yr5; (2) rust incidences in resistant families increased with inoculum density; (3) R1 and R3 were unstable among years; and (4) R2 and R4 were the most resistant and stable family among years (Schmidt et al. 1999). In the current study, R3 was very unstable over time; thus a major difference between the two studies was that in the field R2 was more resistant than R3. Although greenhouse and field studies cannot be compared directly in this study, because different variables were measured (four symptoms in this study, and one symptom in the field studies) and data were taken over a 5-year period in the field, both studies showed family, year, and family x year effects.

Previously, Walkinshaw et al. (1980), Hubbard (1981) and Souza et al. (1991) demonstrated that field performance could be positively correlated with greenhouse data by using the differential gall types and SYMNOS. This Index combines the four-symptom types and ranks the potentially resistant families. When the index was calculated in this study, R2 ranked first in almost all sites and years (Table 2-2). This result agrees with the field data where R2 performed the best in terms of stability and resistance, clearly indicating that smaller galls are an important component in calculating this index.

The results in this study agree with previous work (Jewell and Snow 1972; Kuhlman and Powers 1988; Miller et al. 1976; Powers and Matthews 1979; Walkinshaw 1978); most have suggested small galls indicate resistance. Thus, large gall alone cannot be used to evaluate resistance, especially in predicting field performance from greenhouse tests (artificial inoculations). It also indicates that the index, as a measure of resistance, is an effective indicator of field performance. In addition, it shows that the four symptom types are valuable to evaluate response among resistant families.

Conclusions

The results presented from these artificial inoculations with mixed gall isolates from susceptible families in controlled greenhouse conditions, where inocula were the primary source of variability, indicate that pathogenic variability between years is the primary cause of the family x year interactions.

Although, no significant difference was observed between year isolates across families and locations, significant family by year interactions for PGAL, PSG, and PSY were observed, indicating temporal variation in disease incidence within certain resistant families. For example, for PGAL on family R3 inoculum from Yr2 was more virulent than inoculum from Yr1, while on family R4 inoculum from Yr1 was more virulent than inocula from Yr2.

Overall, considering only PGAL, rust varied significantly among families, ranging from 54% (R3) to 94% (S2). Families were separated into three groups: most resistant, R3; moderately resistant, R1; and susceptible, R4, R2 and S2. However, for PSY and PRO, two groups of families were noted: resistant, R3 and R1; and susceptible, R4, R2 and S2. In contrast, when the Resistance Screening Center index was considered, R2 generally ranked as one of the most resistant families, together with R1 and R3.

A negative correlation between PGAL and PSY was observed, indicating an inverse relationship between the susceptible trait (GAL) and the resistant trait SYMNOS, confirming that SYMNOS is an indicator of resistance.

Although, no significant family x location interaction was observed, indicating that families do not react differentially at different locations when inoculated with a mixture of isolates from susceptible families from different time periods, some geographical differences were found within family R3. Overall the results of this study suggest that the temporal interactions observed in the Rust Virulence field data were primarily the result of pathogenic variability.

CHAPTER 3

VARIATION IN FUSIFORM RUST INCIDENCE ON SELECTED RUST-RESISTANT FAMILIES OF SLASH PINE AND SINGLE GALL ISOLATES OF CRONARTIUM QUERCUUM F. SP. FUSIFORME

Introduction

Fusiform rust, caused by the fungus *Cronartium quercuum* (Miyabe ex Shirai) f. sp. *fusiforme*, is the most important disease of slash pine (*Pinus elliottii* var. *elliottii* Engelm.) and loblolly pine (*P. taeda* L.). Selection and breeding for resistance have substantially reduced fusiform rust incidences (Pye et al. 1995; Schmidt et al. 1981; Snow et al. 1977). However, development and deployment of resistant pines involve some risk, as increases in virulence in fungus populations have occurred where resistant slash pines have been planted (Powers et al. 1977; Snow et al. 1975; Snow et al. 1976). Previous studies suggested the existence of both stable and unstable pine families (Snow et al. 1976), but little is known about the nature of this resistance, especially the stability of rust-resistant pine families and distribution and frequency of virulent and avirulent pathotypes in the field.

Evidence of differential interaction with pine families has been demonstrated by the differential responses of slash pines to inocula throughout the southeastern USA (Snow et al. 1976). Most families were quite variable in responses; some proved susceptible to some inocula and resistant to others. Significant family x isolate interactions were found, suggesting the existence of specific virulent genes among isolates (Dinus et al. 1975;

Powers et al. 1978; Snow et al. 1975). Cross inoculation experiments have shown that *C. quercuum* f. sp. *fusiforme* inocula are usually more infectious on the pine species from which they were collected than were inocula collected from other pine species (Snow et al. 1976). Virulence frequencies of *C. quercuum* f. sp. *fusiforme* varied considerably among the rust collections from different states and among collections within individual states. Some rust collections caused more disease on the family of origin than on others (Powers et al. 1978; Snow et al. 1976; Snow and Griggs 1980). Powers and Matthews (1979) concluded that the relatively high infection levels produced by some family-specific isolates on host families from the same geographic areas indicate that certain rust isolates have adapted to these sources of resistance in the host.

The sexual stage of *C. quercuum* f. sp. *fusiforme*, which occurs on oaks, provides the opportunity for increased genetic variation within the *C. quercuum* f. sp. *fusiforme* population. Based on analysis of RAPD's, Doudrick et. al. (1993a) concluded that a high level of genetic variability exists within populations of this pathogen, with little geographic differentiation. Hamelin et al. (1994), using RAPD markers found evidences of genetic differences in populations east and west of the Mississippi River. Because there is evidence of high genetic variability in both populations of *C. quercuum* f. sp. *fusiforme* and pine species, selection of host-specific, virulent pathotypes adapted to slash pine families in a specific location also may occur. Thus, there is need for more research on the spatial adaptation of *C. quercuum* f. sp. *fusiforme*. This information is a prerequisite for planning breeding programs and deployment of families resistant to *C. quercuum* f. sp. *fusiforme*.

Another difficulty in the breeding program is the interaction of the pine genotypes with the environment. In plantations, rust incidences of resistant slash pine families evaluated in different locations have been reported to be higher than expected and rankings may sometimes be inconsistent (Sohn et al. 1975). Schmidt and Allen (1991), studying the performance of slash pine families in high rust hazard areas, found significant variation among locations. Likewise, Schmidt et al. (1986), studying 1,882 slash and loblolly pine plantations established from 1961 to 1980 in Florida and Georgia, reported that rust incidence increased to the west and north and decreased in the eastern region of slash pine plantations.

The cause of this family with location interaction is still unclear. Also, little is known about the pathogen stability in different locations. Most information is from survey data, which have suggested that location variation in rust incidences was the result of favorable or unfavorable climate for disease development (Schmidt and Allen 1998; Squillace 1976). To better understanding the stability of resistant families, they need to be tested against inocula from field infected members of the same families.

It is difficult to draw conclusions about the spatial stability of resistant slash pine families and isolates of *C. quercuum* f. sp. *fusiforme* because many of these experiments were not repeated at many locations, were done with a limited number of families, or did not use family-specific isolates of *C. quercuum* f. sp. *fusiforme*. Also there are several confounded elements in field studies that could be assigned to the cause of the increased rust incidence in resistant families.

Rust incidence in the field is closely related to climatic, edaphic, and biotic factors (Schmidt et al. 1981; Snow et al. 1977). Ultimately, pine infection depends on the succulence and susceptibility of the new pine growth when spores are present and environmental conditions are favorable for infection and sporulation. Site preparation and fertilization also can result in increased rust incidence (Schmidt et al. 1981). Infections by *C. quercuum* f. sp. *fusiforme* are favored by an abundant period of high relative humidity ($\geq$ 97%), high leaf surface moisture (rain or dew), and temperature from 16 to 27 °C (Dwinell 1977; Schmidt et al. 1981; Snow and Froelich 1981). These conditions are often present in most locations in the high rust hazard areas and usually do not limit rust development in these areas (Schmidt et al. 1981).

In 1987, the Rust Virulence field experiments to characterize the spatial and temporal variability in *C. quercuum* f. sp. *fusiforme* were established in the Coastal Plain of the southeastern United States. Progenies from open- and-control-pollinated, rust-resistant and susceptible slash and loblolly pines families were planted in numerous locations of the coastal plain in 1987, 1989, 1991 and 1993 (Schmidt and Allen 1990). Analyses of the cumulative rust incidence at 5 yr of age in the slash pine plantations examined the performance of slash pine families in high rust hazard locations and demonstrated significant variation in family rust incidence among locations and a family by location interaction. This interaction was the result of a significant difference in rust incidences between families and locations, with rust incidence among locations ranging from 3.6 to 50.8 percent for one family planted in several high rust hazard areas (Schmidt and Allen 1995). This differential response of pine progenies with environment might be a consequence of the different pathotypes that occur in different years and locations. The cause of this interaction cannot be conclusively assigned from the Rust Virulence field studies. Cumulative rust incidences at age 5 years may have been affected by many uncontrolled factors, such as soil, oak abundance, weather, host phenology, or inoculum potential (either quantity or quality of pathotypes).

It is hypothesized that variation in rust incidences among rust collections from different locations might be caused by pathogen variability rather than by climate or phenology. The objective of this research was to study the effect on rust incidence of pathogen isolates collected from four slash pine families (three rust-resistant families and one rust-susceptible family), from four locations in the Rust Virulence field tests. Artificial inoculation under greenhouse conditions at the Resistance Screening Center was employed to control the independent variables present in the field tests, except the quality of inoculum.

Material and Methods

Families

Seeds of four open-pollinated slash pine families represented in the Rust Virulence field tests were used. Their maternal parents were selected in the coastal plain of Florida, and Georgia, and seed orchards were located in the same general area. The parents were chosen from a population previously selected based on their breeding values of rust resistance (R50) and volume growth. An R50 expresses the expected percent of offspring from a given parent that will be infected with rust in an environment in which unimproved material expresses 50% infection. The qualitative ratings of rust resistance, the R50 and volume ratings from the Cooperative Forest Genetics Research Program (CFGRP), at the School of Forest Resource and Conservation (SFRC) at the University of Florida, are given in Table 2-1. Based on these ratings performance, families R1 and R3 were noted as resistant, family R4 as very resistant, and family S2 as very susceptible (Schmidt et al. 1999). Family R2 was not used in this study because there were not enough seeds to conduct this experiment. The performance of these families in four field plantings (13, 17, 19, and 20) in the Rust Virulence study appear in Table 2-2a and 2-2b of Chapter 2.

Isolates

Sixteen collections were chosen representing four locations (13, 17, 19, and 20) and four family-specific isolates at each location (Table 3-1). Each isolate was a single collection from stem galls on trees from each resistant family: R1, R3, and R4, and one from the rust-susceptible family S2. Individual gall collections were chosen since it was believed that these represented isolates from family-specific pathotypes from the surrounding area. The locations were chosen based on (1) yearly variation in the Rust Virulence study for the 1987 and 1989 planting, and (2) high rust-hazard areas. All aeciospore collections were processed separately and stored according to the procedures of Roncadori and Matthews (1966). In brief, the procedures included collection of aeciospores from stem galls using a dual valve suction bulb; screening of aeciospore collections individually through a 50-mesh sieve to remove insects and trash; estimation of germination; and desiccation of aeciospores in ampoules with the aid of a vacuum pump. Ampoules were sealed with a natural gas-air torch, labeled, and stored under refrigeration conditions. Prior to storage, each collection was checked for germination percentage, and assigned a number indicating date, location, and year of infection. Inocula were stored and sent to the Resistance Screening Center, where the experiment was conducted.

Inoculation

To produce basidiospore inoculum, seedlings of northern red oak *(Quercus rubra L.)* were inoculated with aeciospores from each of the 16 collections (four locations- and four family-isolates). Later, basidiospores were harvested from oak leaves and used in a concentrated basidiospore spray system (CBS) for pine inoculations (Knighten et al. 1988; Schmidt et al. 1977). Briefly, pine seeds were germinated, and the seedlings were transplanted immediately after emergence into flats containing 20 seedlings each. At 6 weeks of age, the seedlings were carried on a conveyor belt under a spray of an aqueous suspension of basidiospores. Inocula were adjusted to 20,000 spores per ml. Each of the 16 rust collections was tested on 80 seedlings for each of the four families; thus 5,120 seedlings were inoculated. After inoculation, seedlings were kept in an incubation chamber at 20 °C and 100% relative humidity, for 24 hours. Artificial inoculations were conducted on 2 inoculation days as follow: on each day, families were randomly sprayed with inoculum of each basidiospore collection of *C. quercuum* f. sp. *fusiforme* A mixture of rust-susceptible slash pine families from the Resistance Screening Center was used as a check for the inoculation efficiency.

Six months after inoculation, binary data (presence and absence) of several fusiform rust symptoms were evaluated. The traits were: (1) smooth gall (SO), the gall surface has the same texture as the unaffected stem tissue above and below the swelling; (2) rough gall (RO), the gall surface is rough and reddish-brown with obvious necrotic sunken areas; (3) large gall (LG), gall is more than 25 mm long; (4) small gall (SG), smaller or equal to 25 mm long; (5) purplish discoloration on the stem and no swelling

(SYMNOS); (6) healthy (HE), seedlings with no apparent symptom; and (7) any gall type (GAL).

Experimental Design and Statistical Analyses

The factorial experiment was a split plot in a randomized complete block design with two replications. Whole plots were isolates (4 family-isolates x 4 location-isolates). Families were subplot treatments (Table 3-1).

Analyses of variances were conducted for each group of isolates and symptom traits (Table 3-2). ANOVA was conducted for isolate across locations and across and within families in a pooled analysis. Subsequently the effect of isolates was partitioned into location-isolates and family-isolates, and ANOVA was conducted from each group of isolates across and within families. Inoculation days were considered as random effect and all other factors as fixed. As the interactions of inoculation day with family and inoculation day with family and isolates were not significant, they were pooled with the replication within the year x location x family interaction to generate the subplot error term in Table 3-2. Because the inoculation was conducted into two days, each with two replications, the interactions with days could not be pooled to estimated the whole plot error.

The binomial traits (SYMNOS, GAL, HE, SO, RO, and SG) were analyzed as plot means (percentages). The percentages of seedlings with small galls (PSG), rough galls (PRO), and smooth gall (PSO) were found by dividing the number of plants with these symptoms by the total number of plants with galls. The percentage of seedlings with large gall (PGAL) and SYMNOS (PSY), and healthy plants (PHE), were obtained by dividing the number of plants with these symptoms by the total number of plants. All variables

Table 3-1. Slash pine families and *Cronartium quercuum* f. sp. *fusiforme* isolates employed to study geographical variation in rust incidence in open pollinated slash pine progenies.

	Isolates[w]																
	13				17				19				20				
Family[v]	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**Total**
R1	A[x]	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	1280[y]
R3	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	1280
R4	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	1280
S2	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	1280
Total of Seedlings	320[z]	320	320	320	320	320	320	320	320	320	320	320	320	320	320	320	5120

[v] R1, R2, R3, and R4 represent resistant families; S2 represents a susceptible family.

[w] Each isolate represents inocula from a single gall collected at each location (13, 17, 19, 20) and from specific families:

A is an isolate collected from family R1;
B is an isolate collected from family R3;
C is an isolate collected from family R4;
D is an isolate collected from family S2.

[x] Cells indicate the specific isolates used (for example, 13A= location 13, isolate collected from family R1).

[y] 1280= 2 replications with 20 seedlings each x 2 inoculation days x 16 isolates.

[z] 320=4 families x 2 replications with 20 seedlings each x 2 inoculation days.

Table 3-2. Full model used for analysis of variance (4 families x 2 replicates x 16 isolates x 2 inoculation days to assess geographical variation to *Cronartium quercuum* f. sp. *fusiforme* in resistant slash pine families.

Source of variation	**DF**[q]	**Expected Mean Squares**
Isolates(I)[r]	15	$\sigma^2_e + 8\sigma^2_{DI} + \sum$[s]$(I)$
Family Isolate(Fi)	(3)	$\sigma_e^2 + 8\sigma^2_{FiLiD} + 32\sigma^2_{FiD} + \sum(Fi)$
Location Isolate(Li)	(3)	$\sigma_e^2 + 8\sigma^2_{FiLiD} + 32\sigma^2_{LiD} + \sum(Li)$
Fi*Li	(9)	$\sigma_e^2 + 8\ \sigma^2_{FiLiD} + \sum(Fi*Li)$
Day (D)[t]	1	$\sigma_e^2 + 8\sigma^2_{DI} + 128\ \sigma^2_D$[u]
D*I	15	$\sigma_e^2 + 8\sigma^2_{DI}$[v]
D*Fi	(3)	$\sigma_e^2 + 8\sigma^2_{FiLiD} + 32\sigma^2_{DFi}$[w]
D*Li	(3)	$\sigma_e^2 + 8\sigma^2_{FiLiD} + 32\sigma^2_{LiD}$[x]
D*Fi*Li	(9)	$\sigma_e^2 + 8\sigma^2_{FiLiD}$[y]
Family(F)	3	$\sigma_e^2 + \sum (F)$
F*I	45	$\sigma_e^2 + \sum (F*I)$
F*Fi	(9)	$\sigma_e^2 + \sum (F*Fi)$
F*Li	(9)	$\sigma_e^2 + \sum (F*Li)$
F*Fi*Li	(27)	$\sigma_e^2 + \sum (F)$
Subplot error	176	σ_e^2[z]

[q] Degrees of freedom.

[r] Isolates are year- and location-isolates. Year-isolates are from time periods 1 (1988-89) and 2 (1992-1993); location-isolates are isolates from locations 13, 17, 19, and 20.

[s] $\sum$ represents fixed effect.

[t] Days are inoculations conducted on each of 2 consecutive days.

[u] σ^2_D = estimated variance of inoculation day for a given variable

[v] σ^2_{DI} = estimated variance of inoculation day x isolates x day interaction for a given variable.

[w] σ^2_{DFi} = estimated variance of inoculation day x family-isolates interaction for a given variable.

[x] σ^2_{DLi} = estimated variance of inoculation day x location-isolates interaction for a given variable.

[y] σ^2_{FiLiD} = estimated variance of family- x location-isolates and inoculation day interaction for a given variable.

[z] σ^2_e = estimated variance of year x location x family interaction pooled with the interactions of inoculation day with family and inoculation day with family and isolates (subplot error).

were analyzed as untransformed plot means, since arcsin transformation did not provide improvement in the results. However, only the susceptible variable PGAL, and the resistant variables PRO, PSG, and PSY are presented. The results regarding PHE and PSO are not presented because the former is nearly the inverse of PGAL, and the latter is the inverse of PRO. The results are presented as main effects, and interactions.

The contributions of isolates and family to the family by isolate and family by location-isolate interaction sum of square were calculated according to Mandel (1971). To investigate the pattern of variation in rust incidence for each type of isolate, the sum of squares due to isolates was partitioned into effects of family- and location-isolates, each with three degrees of freedom. Duncan's multiple comparison range test was used to determine if significant differences existed between family-isolates within families and within family-isolates among families. All statistical analyses were conducted with SAS® (SAS Institute Inc. 1989).

Results

Test Means

The overall test means for percent of seedlings with galls (PGAL) were high on both susceptible checks (94.8 % for S2 and 91.0% for the Resistance Screening Center check), indicating successful inoculations.

Among the four variables evaluated, statistically significant interactions ($P \leq 0.05$) were found for: (1) the family x isolate interaction for PGAL, PRO, and PSG; (2) family with family-isolates for PSG; (3) family with location-isolate for PRO; and (4) family with family-isolate with location-isolate for PGAL (Table 3-3). These interactions suggest that families were reacting differentially with the different isolates. The arcsin transformation was evaluated for all variables, but only the trait PSY changed significantly with the transformation, and a significant family with family-isolate interaction ($P < 0.04$) was found. Since the other variables were not affected with the transformation, all statistical tests for PGAL, PSG and PRO were conducted with untransformed data.

Family Effect

The resistant families generally had high mean values for the resistant variables PSY and PSG, and low means for PGAL, and for PRO (Table 3-4). Generally, the differences among slash pine families were highly significant for all variables ($P=0.0001$) (Table 3-3). Thus, the resistant families were different from the susceptible family, except for PRO where family R4 had the same disease incidence as the susceptible family S2 (Table 3-4). Most families considered resistant on the basis of previous tests were also resistant to all isolates in this test.

Based on PGAL, families separated into four groups; R3 was the most resistant (52.6%), followed by R1 (77.8%), R4 (84.8%), and S2 (94.8%) (Table 3-4). Despite the statistically significant differences among families, across all sources of inocula, slash pine resistant families ranged from moderately resistant (R3) to low resistant (R1, R4). On the basis of disease resistant reaction (PSY and PSG) over all isolates, the four slash pine families fell into two distinct groups: families with low incidences of PSY and PSG, S2 and R3; and families with high incidences of PSY and PSG, R1 and R4. Although for PRO, R4 was not statistically different from S2, for the other variables R4 generally was significantly different from the other families; R4 generally had the second highest rust incidence.

Table 3-3. Mean square and significance probability values (within parenthesis) for split plot analysis of variance describing single gall variation in fusiform rust incidences after artificial inoculations with isolates of *Cronartium quercuum* f. sp. *fusiforme* at the Resistance Screening Center.

Sources of variation	**DF**[v]	**PGAL**[x]	**PSY**[x]	**PRO**[x]	**PSG**[x]
Isolates(I)[y]	15	297.02 **(0.02)**[v]	0.007 (0.54)	543.68 (0.07)	245.30 (0.06)
Family Isolate (Fi)	(3)	525.01 (0.14)	0.05 (0.23)	434.27 **(0.04)**	106.15 (0.59)
Location Isolate (Li)	(3)	259.03 (0.26)	0.04 (0.43)	1316.47 **(0.04)**	164.94 (0.33)
Fi*Li	(9)	233.68 (0.07)	0.04 (0.85)	322.56 (0.58)	318.45 **(0.05)**
Day (D)[z]	1	14.06 (0.71)	0.00 (0.98)	5.20 (0.88)	465.37 **(0.05)**
D*I	15	99.68 (0.65)	0.19 (0.08)	253.09 (0.29)	106.52 (0.41)
D*Fi	(3)	126.2 (0.28)	0.02 (0.52)	39.78 (0.95)	141.63 (0.29)
D*Li	(3)	116.20 (0.31)	0.03 (0.32)	114.78 (0.82)	96.54 (0.44)
D*Fi*Li	(9)	85.34 (0.70)	0.02 (0.14)	370.29 (0.08)	98.14 (0.47)
Family(F)	3	20625.5 **(0.0001)**	0.45 **(0.0001)**	7864.43 **(0.0001)**	15699.3 **(0.0001)**
F*I	45	202.84 **(0.01)**	0.15 (0.18)	333.02 **(0.02)**	162.99 **(0.01)**
F*Fi	(9)	124.68 (0.42)	0.03 **(0.04)**	311.39 (0.17)	238.56 **(0.01)**
F*Li	(9)	152.97 (0.26)	0.02 (0.25)	612.5 **(0.004)**	142.77 (0.18)
F*Fi*Li	(27)	245.51 **(0.004)**	0.01 (0.61)	247.07 (0.29)	144.55 (0.09)
Subplot error	176				

[v] Numbers in bold are significant at the 5% level.

[w] Degrees of freedom.

[x] PGAL, PSY, PRO, PSG are the variables used to measure phenotypic responses of slash pine families to *Cronartium quercuum* f. sp. *fusiforme*:

PGAL = percentage of seedlings with any gall type;

PSY = percentage of seedlings showing area of purplish discoloration and no stem swelling;

Table 3-3--continued

PRO = percentages of PGAL seedlings with a gall having an area of discoloration covering more than 50 % of the gall making it rough; and

PSG = percentages of PGAL seedlings with galls smaller or equal to 25 mm long.

[y] Isolates are family- and location-isolates. Family-isolates are collections from a single gall collected from a specific family: A is an isolate collected from family R1; B is an isolate collected from family R3; C is an isolate collected from family R4; D is an isolate collected from family S2. Location-isolates are collections from locations 13, 17, 19, and 20 in Florida and Georgia.

[z] Days are inoculations conducted on each of 2 consecutive days.

Table 3-4. Overall mean percentages of open-pollinated slash pine progenies with four phenotypic responses after artificial inoculation with *Cronartium quercuum* f. sp. *fusiforme*.

	Phenotypic responses [w]							
Families[y]	**PGAL**		**PSY**		**PRO**		**PSG**	
R1	77.8	C[z]	13.4	A	74.0	B	39.9	A
R3	52.6	D	9.2	B	69.9	B	7.9	C
R4	84.8	B	11.6	A	90.8	A	21.6	B
S2	94.8	A	3.3	C	91.2	A	6.1	C

[w] Phenotypic responses of slash pine families to *Cronartium quercuum* f. sp. *fusiforme*:

PGAL = percentage of seedlings with any gall type type;

PSY = percentage of seedlings showing area of purplish discoloration and no stem swelling;

PRO = percentages of PGAL seedlings with a gall having an area discoloration covering more than 50 % of the gall making it rough; and

PSG = percentages of PGAL seedlings with galls smaller or equal to 25 mm.

[y] R1, R3, R4 are resistant families; S2 is a susceptible family.

[z] Values within column followed by the same uppercase letter do not differ significantly according to Duncan's multiple range test (P=0.05).

Isolate Effect

The split-plot analysis of rust incidence for slash pine families and isolates of *C. quercuum* f. sp. *fusiforme* showed that the main effect of isolate was significant only for PGAL ($P \leq 0.05$). All isolates of *C. quercuum* f. sp. *fusiforme* were pathogenic to all families. However, the disease incidence mean across families indicated different patterns among the isolates, suggesting that some isolates were more virulent than others (Figure 3-1). For example, the family-isolate derived from S2 (D) at locations 17, 19, and 20 and derived from R4 (C) at location 19 were more virulent than the isolates derived from the resistant families R3 (B) at locations 19 and 13, and R1 (A) at location 13.

Location-Isolates

Except for PRO, there was no statistically significant main effect of location-isolate across families (Table 3-3), indicating no overall geographic trend among location-isolates. Pairwise comparisons with all four disease variables (PGAL, PRO, PSY, and PSG) (Table 3-5), within families and among locations also did not identify significant across all families and location-isolates location-isolate effect. For example, Incidence ranged from 75% at location 13 to 80% at location 19. Moreover, it was observed that generally the location-isolates were most virulent on S2 and R4 and least virulent on R3. Thus, there was no evidence in these data that isolates from a certain location are significantly more virulent on a particular family, except for PRO, in which a significant variation in virulence was noticed on collections from widely separated areas (Table 3-5).

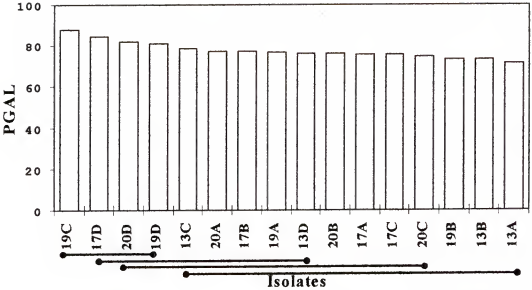

Figure 3-1. Effects of isolates on percentage of seedlings with galls (PGAL) 6 months after inoculation with *Cronartium quercuum* f. sp. *fusiforme*. Four open-pollinated slash pine progenies were inoculated with 16 isolates (4 family-isolates and 4 location-isolates). Means were obtained across progenies. Isolates are family- and location-isolates. Family-isolates are collections from a single gall collected from a specific family: A is an isolate collected from family R1; B is an isolate collected from family R3; C is an isolate collected from family R4; D is an isolate collected from family S2. Location-isolates are collections from locations 13, 17, 19, and 20 in Florida and Georgia. Isolates followed by the same bar line do not differ significantly according to Duncan's multiple range test (P=0.05).

Table 3-5. Phenotypic responses to *Cronartium quercuum* f. sp. *fusiforme* on four open pollinated slash pine progenies 6 months after artificial inoculation.

	Phenotypic responses[v] (%)															
	PGAL				**PSY**				**PRO**				**PSG**			
Fam.[w]	13[x]	17[x]	19[x]	20[x]	13	17	19	20	13	17	19	20	13	17	19	20
R1	76 b[y]	81 b	81 a	73 b	17 a	12 a	10 a	14 a	85 ab	77 a	66 a	69 c	41 a	41 a	34 a	44 a
R3	49 c	49 c	56 b	57 c	8 bc	12 a	7 a	10 ab	80 b	55 b	71 bc	74 bc	8 c	12 bc	5 c	8 c
R4	81 ab	87 ab	84 a	87 ab	14 ab	11 a	9 a	12 a	92 ab	92 a	91 a	89 a	24 b	21 b	19 b	22 b
S2	93 a	97 a	88 a	95 a	5 c	1 b	4 a	3 b	96 a	91 a	89 ab	88 ab	6 c	6 c	8 bc	4 c
Mean[z]	75 A	78 A	80 A	77 A	11 A	9 A	8 A	10 A	88 A	79 B	79 B	80 B	20 A	20 A	17 A	19 A

[v] Phenotypic responses of slash pine families to *Cronartium quercuum* f. sp. *fusiforme*:

PGAL = percentage of seedlings with any gall type type;

PSY = percentage of seedlings showing area of purplish discoloration and no stem swelling;

PRO = percentages of PGAL seedlings with a gall having a of discoloration covering more than 50 % of the gall making it rough; and

PSG = percentages of PGAL seedlings with galls smaller or equal to 25 mm.

[w] R1, R3, R4 are resistant family; S is a susceptible family.

[x] 13, 17, 19, and 20 are location-isolate collections from locations in Florida and Georgia.

[y] Values within columns followed by the lowercase letter do not differ significantly according to Duncan's multiple range test (P=0.05) .

[z] Each value is the mean of four family-isolates (A, B, C, D), two replicates and 2 inoculation days within a location-isolate across all families.

Family-Isolates

Across families, family-isolates were equally virulent. On this basis, differences among family-isolates were not significant, except for PRO (Table 3-3), in which family-isolate A (derived from R1) was the least virulent (77%) (Table 3-6). Resistance to individual family-isolates, however, ranged from moderate to low within each family.

For PGAL, the responses ranged from 48% on R3 when inoculated with family-isolate B to 96% on S2 when inoculated with family-isolate D (Table 3-6). Family-isolate inoculum derived from S2 (D) caused more disease in all families than the other family-isolates. Also, there was no strong evidence that inoculum collected from the other resistant families was more virulent on its own family than were inocula from other families.

Family S2 was remarkably uniform in how it responded to all family-isolates, showing how this family is uniformly susceptible to all four isolates and could be easily separated from the most resistant families.

Interaction Effects

The analysis of variances showed the following significant interactions ($P \leq 0.05$): (1) three-way interaction of family with family-isolate with location-isolate for PGAL; (2) family with isolate interactions for PGAL, PRO, and PSG; (3) family with family-isolates for PSY and PSG, and (4) family with location-isolate interactions for PRO (Table 3-3).

The three-way interaction indicates that percentage of seedlings with galls on a particular family depends on the location- and family-isolate (Table 3-3). This also

Table 3-6. Phenotypic responses on four open pollinated slash pine progenies 6 months after artificial inoculation with family-isolates of *Cronartium quercuum* f. sp. *fusiforme*.

	Phenotypic responses[u] (%)											
	PGAL				PRO				PSG			
Families[v]	A[w]	B	C	D	A	B	C	D	A	B	C	D
R1	$\underline{75}^{x}$ c^{y}	77 bc	80 b	80 b	$\underline{71}$ b	78 b	74 b	73 b	$\underline{47}$ a	36 a	38 a	38 a
R3	49 b	$\underline{48}$ d	53 c	60 c	60 c	$\underline{79}$ b	71 b	70 b	4 c	$\underline{10}$ c	8 c	10 c
R4	85 a	79 b	$\underline{88}$ ab	87 b	91 a	89 a	$\underline{93}$ a	91 a	20 b	27 b	$\underline{20}$ b	19 b
S2	92 a	96 a	95 a	$\underline{96}$ a	89 a	88 a	94 a	$\underline{94}$ a	7 c	8 c	7 c	$\underline{2}$ c
Mean[z]	75 A	75 A	79. A	81 A	77 B	83 A	83 A	82 A	19 A	20 A	18 A	17 A

[u] Phenotypic responses of slash pine families to *Cronartium quercuum* f. sp. *fusiforme*:

PGAL = percentage of seedlings with any gall type;

PSY = percentage of seedlings showing area of purplish discoloration and no stem swelling;

PRO = percentages of PGAL seedlings with a gall having a of discoloration covering more than 50 % of the gall making it rough; and

PSG = percentages of PGAL seedlings with galls smaller or equal to 25 mm.

[v] R1, R3, R4 are resistant families; S2 is a susceptible family.

[w] Family-isolates are collections from a single gall collected from a specific family: A is an isolate collected from family R1; B is an isolate collected from family R3; C is an isolate collected from family R4; D is an isolate collected from family S2. Location-isolates are collections from locations 13, 17, 19, and 20 in Florida and Georgia.

[x] Underline figures represent seedlings inoculated with inoculum derived from that family.

[y] Values within column followed by the same lowercase letter do not differ significantly according to Duncan's multiple range test (P=0.05).

[z] Each value is the mean of two replicates and 2 inoculation days within a family-isolate (A, B, C, D)

indicates pathogenic variability among isolates from the same locations; major emphasis is placed on the outcome of analyses involving only this interaction. To assist in interpreting the tree-way interaction, the mean of family-isolates was computed for each "cell" in the experimental design. Generally, comparisons within locations showed significant differences among and within families (Table 3-7). These differences were significant for all locations ($P < 0.05$). Substantial variability in the response of R3 was observed in all locations, and within location among family-isolates.

Although no significant differences in rust incidence were found among family-isolates, comparisons within a location and within a family showed that the ranking of family-isolates differed in some cases depending upon which family they were tested on. For example, within location 13 and family R3, the family-isolate derived from family R4 (C) was significantly more virulent on family R3 than the other isolates, whereas within family R4 the least virulent family-isolate was that derived from family R3 (B) (Table 3-7). Moreover, no differences among family-isolates were detected within families R1 and S2. To better understand the importance of these interactions, graphs were plotted for all variables (Figure 3-2 to 3-5). Although families responded differentially, depending on which isolates they were tested on, based on PGAL and within family R3, the reaction pattern of the 16 isolates on four slash pine families indicated that isolates that came from the susceptible S2 (D) at location 20 and from the resistant R4 (C) at location 19 were the most virulent for the variable PGAL (Figure 3-2); whereas for the other variables no clear pattern was observed (Figure 3-2 to 3-4). In contrast, no differential responses to isolates were observed for S2 in any variable (Table 3-2 to 3-5). Analysis of the family contribution to these interactions showed that the variation involved was primarily due to

Table 3-7. Effects of location and family isolates of *Cronartium quercuum* f. sp. *fusiforme* on percentage of seedlings with galls (PGAL) on four open pollinated slash pine progenies.

	Percentage Gall Incidence [v](%)															
	13 [w]				17				19				20			
Fam [u]	A[x]	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
R1	64 Ab[y]	84 Aab	76 ABab	83 Aa	77 Ab	81 Ab	82 Aa	84 Aab	80 Aa	74 Ab	86 Aab	85 Aa	77 Aa	72 Ab	75 Ab	68 Ac
R3	41 Bc	46 Bc	62 Ac	45 Bb	48 Bc	47 Bc	33 Cb	70 Ab	53 Bb	45 Bc	74 Ab	51 Bb	56 Bb	53 Bc	45 Bc	76 Abc
R4	85 Aa	70 Bb	84 Aab	85 Aa	85 Aab	86 Aab	89 Aa	88 Aab	84 ABa	81 Bab	98 Aa	89 ABa	85 Aa	83 Aab	84 Aab	87 Aab
S2	96 Aa	93 Aa	93 Aa	93 Aa	94 Aa	98 Aa	98 Aa	98 Aa	89 Aa	96 Aa	94 Aa	99 Aa	91 Aa	98 Aa	95 Aa	96 Aa
Mean [z]	72A	73AB	79B	76AB	76A	78A	75A	85A	76BC	74C	88A	81B	77A	76A	75A	82A

[u] R1, R3, R4 are resistant family; S2 is a susceptible family.

[v] Percentage of seedlings with any gall type.

[w] 13, 17, 19, and 20 are location-isolates collections from locations in Florida and Georgia.

[x] Family-isolates are collections from a single gall collected from a specific family: A is an isolate collected from family R1; B is an isolate collected from family R3; C is an isolate collected from family R4; D is an isolate collected from family S2. Location-isolates are collections from locations 13, 17, 19, and 20 in Florida and Georgia.

[y] Values within column followed by the same lowercase letter and within a row followed by the same uppercase letter do not differ significantly according to Duncan's multiple range test (P=0.05).

[z] Each value is the mean of two replicates and 2 inoculation days within a location-isolate (13, 17, 19,20) and family-isolate (A, B, C, D) across all families.

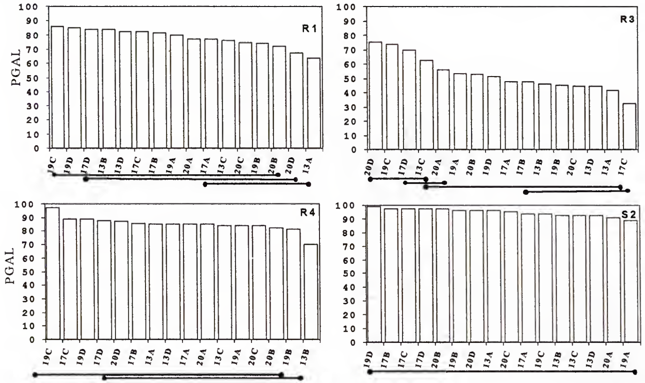

Figure 3-2. Family by isolate (location- and family-isolate) interaction on percentage of seedlings with galls (PGAL) on progeny (R1, R3, R4, S2) of open-pollinated slash pine progenies 6 months after artificial inoculations with *Cronartium quercuum* f. sp. ***fusiforme*.** Family-isolates are collections from a single gall collected from a specific family: A is an isolate collected from family R1; B is an isolate collected from family R3; C is an isolate collected from family R4; D is an isolate collected from family S2. Location-isolates are collections from locations 13, 17, 19, and 20 in Florida and Georgia. Isolates followed by the same bar line do not differ significantly according to Duncan's multiple range test (P=0.05).

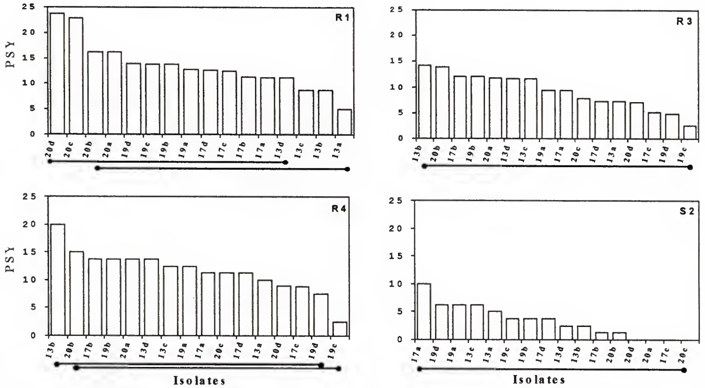

Figure 3-3. Family by isolate (location and family isolate) interaction on percentage of seedlings with symptom and no swelling (PSY) on open-pollinated slash pine progenies six months after artificial inoculations with *Cronartium quercuum* f. sp. *fusiforme*. Isolates are family- and location-isolates. Family-isolates are collections from a single gall collected from specific families: A is an isolate collected from family R1; B is an isolate collected from family R3; C is an isolate collected from family R4; D is an isolate collected from family S2. Location-isolates are collections from locations 13, 17, 19, and 20 in Florida and Georgia. Isolates followed by the same bar line do not differ significantly according to Duncan's multiple range test (P=0.05).

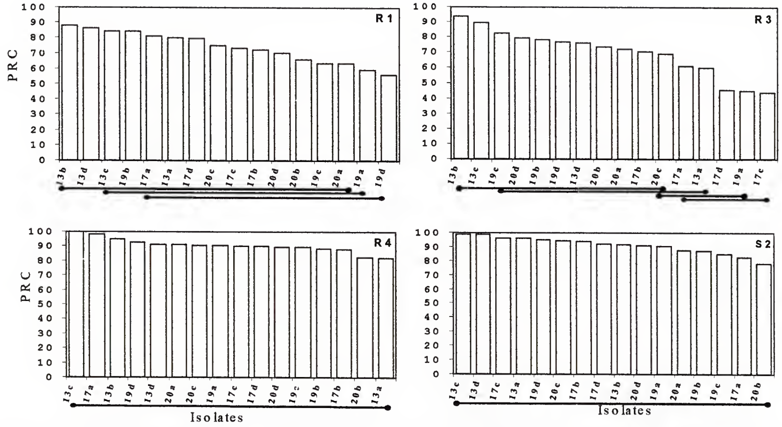

Figure 3-4. Family by isolate (location- and family- isolate) interaction on percentage of seedlings with rough galls (PRO) on progeny (R1, R3, R4, S2) of open-pollinated slash pine progenies 6 months after artificial inoculations with *Cronartium quercuum* f. sp. *fusiforme*. Family-isolates are collections from a single gall collected from a specific family: A is an isolate collected from family R1; B is an isolate collected from family R3; C is an isolate collected from family R4; D is an isolate collected from family S2. Location-isolates are collections from locations 13, 17, 19, and 20 in Florida and Georgia. Isolates followed by the same bar line do not differ significantly according to Duncan's multiple range test (P=0.05).

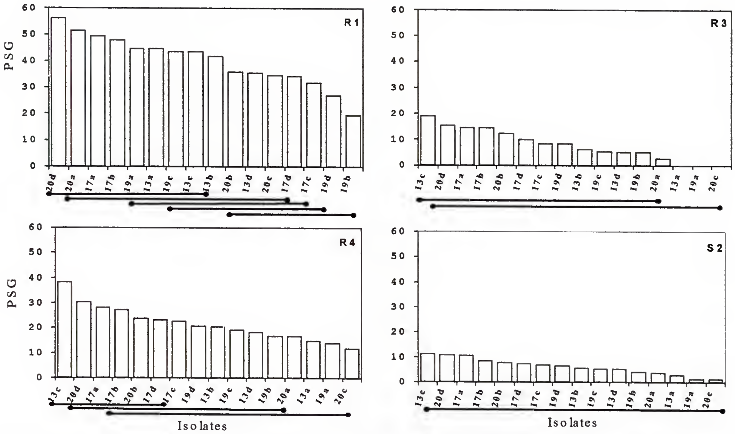

Figure 3-5. Family by isolate (location- and family- isolate) interaction on percentage of seedlings with short galls (PSG) on progeny (R1, R3, R4, S2) of open-pollinated slash pine progenies 6 months after artificial inoculations with *Cronartium quercuum* f. sp. *fusiforme*. Family-isolates are collections from a single gall collected from a specific family: A is an isolate collected from family R1; B is an isolate collected from family R3; C is an isolate collected from family R4; D is an isolate collected from family S2. Location-isolates are collections from locations 13, 17, 19, and 20 in Florida and Georgia. Isolates followed by the same bar line do not differ significantly according to Duncan's multiple range test (P=0.05).

R3, except for PSG in which R1 contributed more to the family-by-family-isolate (39.8%) and family-by - isolate interactions (31.9%) (Table 3-8). The differential response of slash pine families to family and location-isolates is an indication of the differential pathogenicity of *C. quercuum* f. sp. *fusiforme* among slash pine families.

There was a correlation of r = - 0.83 (P=0.0001) between the field prediction index for rust resistance and the mean field disease incidence (MINC) over 3 planting years (Table 3-9). Of special interest is the fact that there was an indirect relationship between PGAL in the field and the index at the greenhouse (R^2=0.69) (Figure 3-6). As shown in Figure 3-6, higher index values corresponded to lower field disease incidence (PGAL); clearly the susceptible family had the highest field disease incidence at the lowest index values.

The relatively strong correlation between these two variables suggests that the index coefficient provides accurate estimates of rust resistance on an open-pollinated family, particularly for susceptible or very resistant families.

These comparisons indicate that large gall alone cannot be used to evaluate resistance, especially in predicting field performance from greenhouse tests (artificial inoculations). It also indicates that the index as a measure of resistance, is an effective indicator of field performance. In addition, it shows that the four symptom types are valuable to evaluate response among resistant families.

Table 3-8. Percentage contribution of slash pine open pollinated progenies to interaction of (A) family with family-isolate, (B) family with location-isolate, and (C) family with isolate interactions 6 months after artificial inoculation with *Cronartium quercuum* f. sp. *fusiforme*.

		Percentage of variation [v]			
Source of variation	**Fam** [x]	**PGAL**	**PSY**	**PRO**	**PSG**
A) Family by family-isolate [y]					
	R1	8.9	9.1	5.0	**39.8**
	R3	**34.6**	22.9	**49.9**	33.9
	R4	28.39	23.2	15.9	7.8
	S2	28.0	**47.7**	29.1	18.3
B) Family by location-isolate [z]					
	R1	38.3	8.1	17.8	24.9
	R3	**47.9**	**45.8**	**64.6**	25.7
	R4	2.8	3.6	14.2	5.6
	S2	11.0	42.6	3.4	**43.7**
C) Family by isolate					
	R1	23.9	24.8	18.5	**31.9**
	R3	**50.4**	29.5	**46.2**	27.4
	R4	10.2	14.0	12.9	18.9
	S2	15.5	**31.7**	22.3	21.7

[v] Percentage of variation of slash pine families to *Cronartium quercuum* f. sp. *fusiforme* (values in bold represent the highest family contribution to the interaction):

PGAL = percentage of seedlings with any gall type;
PSY = percentage of seedlings showing an area of purplish discoloration and no stem swelling;
PRO = percentages of PGAL seedlings with a gall having an area of discoloration covering more than 50 % of the gall making it rough; and
PSG = percentages of seedlings with galls smaller or equal to 25 mm long.

[x] R1, R3, and R4 represent resistant families; S2 represents a susceptible family.

[y] Family-isolates are collections from a single gall collected from a specific family: A is an isolate collected from family R1; B is an isolate collected from family R3; C is an isolate collected from family R4; D is an isolate collected from family S2.

[z] Location-isolates are collections from locations 13, 17, 19, and 20 in Florida and Georgia.

Table 3-9. Correlation Coefficients between field disease incidences based on percentage of seedlings with any gall type (PGAL) and Index values at the Resistance Screening Center.

	PGALGH[v]	MINC[w]	Plantation[x] 87	R/S[y] 87	Plantation 89	R/S 89	Plantation 91	R/S 91
INDEX[z]	-0.22	**-0.83**	**-0.83**	-0.41	**-0.87**	-0.12	**-0.91**	-0.29
P value	(0.407)	**(0.0001)**	**(0.0001)**	(0.1902)	**(0.0001)**	(0.7357)	**(0.0001)**	(0.4488)
PGALGH[v]		**0.53**	0.49	-0.21	-0.53	-0.23	0.51	-0.48
P value		**(0.0348)**	(0.0564)	(0.5215)	(0.0607)	(0.53)	(0.0739)	(0.194)
MINC[w]			**0.97**	**0.80**	**0.96**	0.60	**0.94**	0.63
P value			**(0.0001)**	**(0.0018)**	**(0.0001)**	(0.0681)	**(0.0001)**	(0.0719)

[v] PGALGH = percentage of seedlings with any stem swelling in greenhouse tests.

[w] MINC = mean of field disease incidence (PGAL) over the 3 planting years.

[x] Plantation represents the year which a plantation was first established. For example, in plantation 1, trees were planted in 1987; in plantation 2, trees were planted in 1989; in plantation 3, trees were planted in 1991.

[y] R/S represents the ratio of proportion of trees infected with fusiform rust on resistant (R) open-pollinated slash pine families to that on the average proportion of trees infected on two susceptible (S) open-pollinated slash pine families at age 5 yr.

[z] Index (I) represents a value used to predict field performance of slash pine families to fusiform rust resistance.:

$I= 25[PSY - M_1/S_1 + PRO - M_2/S_2 + PSG - M_3/S_3 + PHE - M_4/S_4]$, where

S= standard deviation for each symptom;

M= minimum value for each symptom over families;

PSY = percentage of seedlings showing an area of purplish discoloration and no stem swelling;

PRO = percentages of PGAL seedlings with a gall having an area of discoloration covering more than 50 % of the gall making it rough;

PSG = percentages of seedlings with galls smaller or equal to 25 mm long; and

PHE= percentages of seedlings with no apparent symptom.

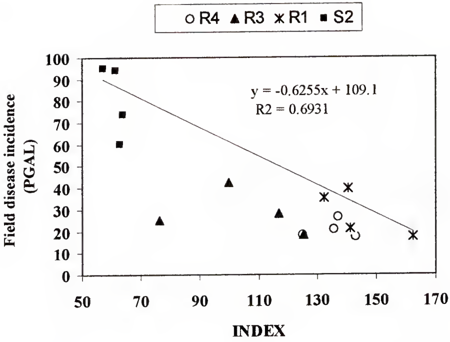

Figure 3-6. Comparisons of field prediction index for rust resistance with field disease incidence based on percentage of seedlings with any gall type for four open-pollinated slash pine progenies 6 months after inoculation with *Cronartium quercuum* f. sp. *fusiforme* at the Resistance Screening Center (RSC); R1, R3, R3 are resistant families, S2 is a susceptible family.

Discussion

Artificial Inoculation

Although artificial inoculation in the greenhouse was employed to control the potential causal factors of the family x location interaction present in the field trials, variability was still evident in the greenhouse data. The inocula used herein represented a measurement of the variation in virulence within isolates from individual galls on four open-pollinated progenies previously classified as rust-resistant and rust-susceptible. Inocula was collected from four family-specific isolates at four locations in high hazard areas, to evaluate variation among the 16 collections. Although a gall may be the product of a single haploid basidiospore, it likely was initiated by more than one basidiospore. Thus, even within a single-gall isolate, many pathogen genotypes may contribute toward the resistance of specific families. Therefore, there is a high probability for heterogeneity in an aeciospore collection from a single gall, as was demonstrated by several workers (Doudrick et al. 1993b; Hamelin et al. 1994; Powers 1980; Powers et al. 1977).

Another source of variability is the open-pollinated slash pine families. The likely heterogeneity of *C. quercuum* f. sp. *fusiforme* inoculum and the heterozygosity in open-pollinated families are likely to have influenced levels of disease. The problem of limiting heterogeneity in the host and in *C. quercuum* f. sp. *fusiforme* have long been challenging, and can best be accomplished with single-spore fungal cultures and clonal host material.

The differences in the amounts of disease in some of the three resistance families was greater than expected. The high disease incidences that occurred on some of the resistant families might have been the result of several factors. It could have been the result of high inoculum density used in this study, as some resistant families did not separate from the susceptible family. Also, under greenhouse conditions the timing of biological events, such as phenology (susceptible, succulent tissue) and basidiospore availability, are more favorable for infection than in field conditions.

The main goals of this study were to determine whether there was variation in the rust population of *C. quercuum* f. sp. *fusiforme* and to investigate the cause of the family x location interaction identified in the field studies. It is important to find the causes of this interaction because it is a key in formulating breeding and deployment programs for disease resistance. Many studies have reported differences in virulence among isolates of this pathogen, as indicated by a family x isolate interaction for disease incidence (Griggs and Walkinshaw 1982; Kuhlman et al. 1990; Powers et al. 1978; Snow et al. 1976). Other studies have also identified location and family x location effects in field or laboratory studies (Anderson and Walkinshaw 1989; Goddard and Schmidt 1979; Powers et al. 1977; Snow and Griggs 1980). The results in this study showed highly significant family x isolate and family x family-isolate x location-isolate interactions for PGAL. The interaction of rust incidences among families with inocula from different locations and families indicated that: (1) some families were not stable across locations, (2) isolates of the pathogen varied in the levels of disease they caused, and (3) the ranking of families depended on the isolate (family- and location-isolate) that they were tested on. Because of the presence of the three-way interaction, it is important to examine the effects of each isolate on each family. Comparison analyses over families indicated that PGAL was low at location 13, when inoculated with family-isolate A, but high at location 19 when family-isolate D was used. Some families were quite stable in response; they proved susceptible to all inocula, whereas one family R3, was quite resistance to inoculum from location 17 and location 13, when inoculated with family-isolates C and A (33% and 41%), and moderately resistance to inoculum from location 19, when inoculated with family-isolate C (74%).These data suggest that families may react differentially to inoculum from different single-gall isolates.

There was no significant effect of location-isolate. Several factors, could have contributed to this result. It is likely that this result was confounded by variation in inoculum (family-isolates), as the averages (four family-isolates within location) resemble composites of individual galls within locations. Perhaps selection operating on specific fungal genotypes was not strong enough to cause significant shifts in the genetic structure of the pathogen population during this time period. Finally, it is possible that *C. quercuum* f. sp. *fusiforme* is a highly heterogeneous pathogen with little geographic variation, as indicated by Hamelin et al. (1994).

Significant variation in disease incidence caused by single-gall isolates of *C. quercuum* f. sp. *fusiforme* is primarily that of gall to gall variation in pathogenicity. This is supported by the statistical significance of the main effect of isolates, and interactions of family with isolates, and family with family- and location-isolates for PGAL (Table 3-3). Since the family by family-isolates and family by location-isolates was not significant, no family- or location-isolate was more virulent across all families. However, generally individual isolates from different locations or from different families varied significantly for PGAL (Figure 3-2) and for PRO (Figure 3-4). For example, for PGAL and within family R3, inoculum from location 20 derived from the susceptible family (D) was more virulent than inoculum from location 17 derived from family R4 (C) (Figure 3-2).

Disease incidences on three of these resistant families, averaged across all isolates, were moderate to high. Disease incidence on R3 was 53.3%, whereas families R1 and R4 exhibited 77.8 and 84.8 % disease incidence, respectively. The significant variation in rust incidences among families was expected, since families were previously selected for their levels of resistance. However, family means across isolates indicated that there was differential resistance among families. Percentage of galls ranged from 52.6% to 94.8%, with R3 being generally the most resistance for all variables, except PSG (Table 3-4). Typically, families separated into three groups: most resistance, R3; moderately resistance, R1; and susceptible, R4 and S2. The data indicated that family R4, R1 were relatively stable across rust isolates, while the most resistant family R3, was most unstable across location and family-isolates. The resistance of families, with such instability can be overcome by extant virulent forms or by future shifts in virulence of the rust population. Apparently, the genetic bases for resistance differ among these three, open-pollinated, rust-resistance families, as suggested by others (Kuhlman et al. 1995; Miller et al. 1976; Schmidt and Miller 1999).

On the basis of symptom expression, the frequency of SYMNOS varied the least (from 8 to 10%); with the highest and the lowest frequency of SYMNOS occurring in families with low and high frequencies of galls; respectively (Table 3-5). Conversely, comparable data concerning symptom expression among open-pollinated slash pine progenies after inoculation with *C. quercuum* f. sp. *fusiforme* (Kuhlman and Powers 1991) indicated that both the highest and the lowest frequency of SYMNOS occurred in families with low frequency of galls. The variation in SYMNOS frequency was attributed to the heterozygosity in the open-pollinated material. Further, regression analysis indicated that increasing gall frequency and decreasing frequency of the resistant symptoms SG, RO, and SYMNOS reflected increasing susceptibility (Figure 3-6). Thus, the observed variation between the two groups of families indicates that gall types and SYMNOS can indeed be a resistant reaction as previously suggested by Miller et al. (1976).

These results support other researchers who found family and family x location effects in field or laboratory studies (Goddard and Schmidt 1979; Snow et al. 1975; Snow and Kais 1970; Sohn et al. 1975); most workers suggested that the primary cause of these effects was pathogen variability. However, in field studies, it could not be confirmed (Sohn and Goddard 1979; Goddard and Schmidt 1979; Schmidt and Allen 1997). These authors have suggested that a location effect may have occurred due to the effect of a climate on inoculum production or pine phenology. In this study, the fact that differences between the inoculum sources were detected within some locations is of primary importance because it allows for the possibility that *C. quercuum* f. sp. *fusiforme* may shift in virulence. Also, it provides strong evidence that pathogenic variability is the cause of the family x family-isolate x location-interaction.

Comparisons with Rust Virulence Field Data

The greenhouse data support results of the Rust Virulence study. Briefly, the Rust Virulence study showed that: (1) there were statistically significant location effects; (2) rust incidences in resistant families increased with inoculum density; (3) R1 and R3 were relatively unstable (Schmidt and Allen 1997; Schmidt and Allen 1998; Schmidt et al. 1999). In the current study, R3 was the most resistant and unstable family across isolates (location-isolate and family-isolate); thus a major difference between the two studies was that in the field R1 and R2 was more resistant than R3. Family R2 was not used in this artificial inoculation study because there were not enough seeds to conduct this experiment. Field studies cannot be comparable directly because different variables were measured (four symptoms in this study, and one symptom in the field studies) and in the field study data were taken over a 5-year period. However, both studies indicated that families are reacting differently to specific isolates at numerous locations. Also, basidiospore inocula for the greenhouse studies were produced on *Q. rubra*, and basidiospore inocula in the field studies came from several oak species, but primarily water oak (*Q. nigra*) and laurel oak (*Q. larifolia*). Dwinell (1985) has shown differences in susceptibility among these oak species. Previously, Walkinshaw et al. (1980), Souza et al. (1991) and Hubbard (1981) demonstrated that field performance could be positively correlated with greenhouse data by using the differential gall types and SYMNOS. This Index combines the four-symptom types and ranks the potential resistant families. When the index was calculated in this study, R1 ranked first in almost all locations (Figure 3-6). This result agrees with the field data, where R1 performed well in terms of stability and resistance.

The results in this study agree with previous work that suggested small galls as indicative of resistance (Jewell and Snow 1972; Powers et al. 1978). It shows that large galls alone cannot be used to evaluate resistance, especially in predicting field performance from greenhouse tests (artificial inoculations). It also indicates that the index as a measure of resistance is an effective indicator of field performance. In addition, it shows that the four symptom types are valuable for evaluating response among resistant families, and should not be ignored.

Conclusions

The results presented from these artificial inoculations under controlled greenhouse conditions, where inocula were the primarily source of variability, indicate that pathogenic variability was the cause of the family x family-isolate x location-isolate interaction.

Although no significant variation was observed between location-isolates across families and family-isolates, a significant family x family-isolate x location-isolate interaction for PGAL was observed, indicating that spatial variation occurred within certain resistant families. Further, isolates of the pathogen varied in the incidence of disease they caused, and ranking of families for resistance depended on the isolate they were tested with.

Some families were quite stable in response; they proved susceptible to all inocula. Other families, family R3 for example, was quite resistance to inocula from location 17 and location 13, when inoculated with family-isolate C and A (33% and 41% disease), and moderately resistance to inocula from location 19, when inoculated with family-isolate C (74%).

Overall, considering only PGAL, rust varied significantly among families. Disease incidences averaged across all isolates were R3, 52.6%; R1, 77.8%; R4, 84.8%, and S2, 94.8%. Families R4 and S2 were generally equally susceptible to all isolates.

A negative correlation of -0.83 (P=0.0001) was found between the field prediction index (where higher index values indicate greater resistance) for rust resistance and the mean field disease incidence over 3 planting years. The relatively strong correlation between these two variables suggests that the index provides accurate estimates of rust resistance on an open-pollinated family, particularly for very susceptible or very resistant families. A negative correlation between PGAL and PSY was also observed, indicating a inverse relationship between the susceptible trait, GAL, and the resistant trait, SYMNOS, therefore confirming the trait SYMNOS as an indicator of resistance.

Overall the results of this study suggest that the family by location interaction observed in the Rust Virulence field data was likely the result of pathogenic variability.

CHAPTER 4

INFLUENCE OF FAMILY, GALL TYPE, AND PATHOGEN ISOLATES ON SPORULATION OF CRONARTIUM QUERCUUM F. SP. FUSIFORME ON SELECTED RUST-RESISTANT FAMILIES OF SLASH PINE

Introduction

Fusiform rust of southern pines, caused by the fungus *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*, is a highly destructive and commercially important disease of slash pine (*Pinus elliottii* (Engelm) var. *elliottii*) and loblolly pine (*Pinus taeda* L.) (Powers 1975; Pye et al. 1997). The pathogen is a heteroecious, macrocyclic fungus, with spermatial and aecial stages produced on pine, and basidiospores produced on *Quercus* spp. (red oak group) (Czabator 1971).

Variation in symptom expression to *C. quercuum* f. sp. *fusiforme* among families of several pine species has been given as evidence for variation in mechanisms of resistance on seedlings of selected pine families 6-12 months after inoculation (Griggs 1985). Symptoms typically evaluated are the presence, shape, and size of galls and red to purplish lesion on the stem with no swelling (Hubbard 1981; Walkinshaw et al. 1980). Miller et al. (1976) observed that families expressing a purplish lesion on the stem, resembling a hypersensitive reaction, did not develop galls. Walkinshaw et al. (1980) and Hubbard (1981) evaluated four indices for predicting relative field resistance and showed that the four symptom types, short gall, purplish lesions on the stem with no swelling, smooth gall, and rough gall, could be used to predict relative levels of rust resistance of pine families. Since then, gall shape and appearance are used as further indications of resistance and susceptibility to fusiform rust. This method allowed the correlation of greenhouse traits to resistance in field tests; it also allowed the development of a selection index predictive of family rust resistance under field conditions.

Although efforts have been made to quantify the size and shape of galls as a further indication of resistance, disease development in field trials is often scored as the percentage of trees with any gall. Griggs et al. (1982), studying short and large galls, found that slash pine seedlings expressing short galls limited colonization by the rust fungus, whereas seedlings with large galls were extensively colonized. Snow et al. (1990) suggested that round galls (usually short galls) on loblolly pine seedlings indicated restricted pathogen development and, therefore, more host resistance than seedlings with large galls. It has been suggested that the tendency of some slash pine families to develop small galls is controlled by the genotypes of both the host and the pathogen (Dinus 1969; Powers 1980; Schmidt and Miller 1999a).

There is also evidence that some galls do not sporulate or sporulate at low frequencies in the field. Kuhlman (1981), observing sporulation on live galls in South Carolina and North Carolina, found that rust galls on slash and loblolly pines may produce both aecia and spermatia, or only spermatia or aecia. He reported that 44-77% of galls produced only aeciospores, and 19% produced only spermatia. Similarly, in northern Florida and southern Georgia, 51% of the galls produced aeciospores during two seasons, 11% produced aeciospores every year, 4% every other year, 25% only once in 4 years, and 18% never sporulated (Chappelka and Schmidt 1984). Aeciospore initiation in Florida was correlated with average minimum January temperature (Chappelka and Schmidt

1983). Later, Kuhlman and Matthews (1985) reported that pine host and rust sources affected spermatial and aecial sporulation. Schmidt and Miller (1999b), studying the influence of inoculum concentration on sporulation of large galls on slash and loblolly pine seedlings, observed that percentages of seedlings showing only spermatia varied inversely with inoculum concentration, whereas the percentages of seedlings showing only aeciospores or aeciospores and spermatia varied directly with inoculum concentration. They suggested this could be due to host specificity. Schmidt and Miller (1999a) also found that a resistant family expressing a high frequency of short galls sporulated more infrequently than other less resistant and susceptible families.

In many plant species loss of major gene resistance has been observed (Johnson 1981). Therefore, more durable types of resistance have been sought. This is particularly important for perennial crops. One type of resistance that has been shown durable is that associated with reduction or absence of sporulation. In the fusiform rust pathosystem, few studies have characterized the pattern of sporulation of families presenting different degrees of resistance, as for example different reaction types. This study was designed to evaluate variation in sporulation on resistant, open-pollinated slash pine families expressing large galls and short galls after inoculation with different isolates of *C. quercuum* f. sp. *fusiforme*.

Material and Methods

The plant material and fungal isolates used in this study were samples from the geographical variation study presented in Chapter 3. The complete description of the selection procedures for families, isolates, aeciospore collection and inoculation technique are described in Chapter 3. A brief description is presented herein.

Families

Seeds of four, open-pollinated, slash pine families, three rust-resistant (R1, R3, R4) and one rust-susceptible (S2), represented in the Rust Virulence field tests, were used. The parents were chosen from a population previously selected based on their breeding values for rust resistance (R50) and volume growth. The quantitative ratings of rust resistance, for R50 and volume are presented in Table 2-1. The performances of these families in four field plantings (13, 17,19, and 20) in the Rust Virulence study appear in Table 2-2a and 2-2b.

Isolates

Sixteen, single-gall rust collections, representing four location-isolates (13, 17, 19, and 20) and four family-isolates (A, B, C, D) at each location were tested on 80 seedlings of each of the four families in this study. Each isolate was a single collection of aeciospores from a stem gall from each of the resistant families R1, R3, and R4, and one was from the rust-susceptible family S2. Individual gall collections were chosen since it was believed that these represented isolates from family-specific pathotypes from the surrounding area.

Inoculation

Six-week-old seedlings were inoculated at the Resistance Screening Center with basidiospores obtained from telia on northern red oak *(Quercus rubra* L.), which had been inoculated with each of the 16 single-gall collections of aeciospores by the technique of Matthews and Rowan (1972). Eighty seedlings per family were inoculated. The inoculation procedure has been briefly described in Chapter 3. The concentration of the inoculum was adjusted to 20,000 spores per ml. After inoculation, seedlings were kept in an incubation chamber at 20 °C and 100% relative humidity for 24 hours. Then, they were grown in a greenhouse for 12 months. A mixture of rust-susceptible slash pine families from the Resistance Screening Center was used as a check for the inoculation efficiency.

Twelve months after inoculation, five seedlings per family, each expressing large galls (typical fusiform rust symptom) or small galls (resistant symptom), were randomly selected to evaluate sporulation. Families separated by isolates and gall types were brought to the greenhouse at the School of Forest Resource and Conservation, Gainesville, where the sporulation data were collected. Trees were not selected for any characteristics except gall type.

Sporulation Assessment

For the purpose of this study, it was assumed that each spermatial droplet on the gall came from one spermagonium. Data were taken for sporulation frequency and sporulation capacity. *Sporulation frequency* (SF) was recorded as the percentage of trees with spermatial droplets (spermatial frequency) only, with aeciospores (aecial frequency) only, or with both spermatia droplets and aeciospores. *Sporulation capacity* (SC) (commonly refers to severity in terms of disease) was recorded as the proportion of gall surface area covered by a given spore type, spermatia or aeciospores, termed spermatial and aecial capacity, respectively. To obtain spermatial capacity, the proportion of gall surface area covered by spermatial exudate was assessed by counting the number of spermatial droplets on the stem and estimating their sizes. The surface gall area for each gall was estimated as length (cm) * width (cm) * 0.9 (correction factor). The correction factor was estimated using a computer scanner method, based on 15 randomly collected large galls and 15 short galls (Cowling and Horsfall 1990). The proportion of area covered by spermatia exudate relative to the surface gall area was used as an estimate of spermatial capacity. Aecial capacity was the proportion of gall surface area covered by aeciospores using a pictorial scale (James 1971). *Sporulation potential (SP)* of a family represents the surface area of a gall that produced spermatia or aecisopores. It was computed for each family and each spore type (spermatia and aeciospores) as

$$SP_i = P_{LG} * SF_{LG} * A_{LG} * SG_{LG} + P_{SG} * SF_{SG} * A_{SG} * SG_{SG}$$

where

SP_i is the sporulation potential of i^{th} family expressed in cm^2 per tree;

P_{LG} and P_{SG} are the proportions of trees with large galls and short galls, respectively;

SF_{LG} and S_{FSG} are the proportions of sporulation trees with large galls and short galls, respectively;

A_{LG} and A_{SG} are the surface areas of large and short galls, respectively; and

SG_{LG} and SG_{SG} are the maximum proportions of a gall surface area covered by a spore type on large and short galls, respectively.

Experimental Design and Statistical Analyses

The factorial experiment was in a complete randomized design with five replications (single tree plots). ANOVA was conducted for isolates across locations and across and within families in a pooled analysis. Duncan's multiple range test was used to determine if significant differences existed between family-isolates within families and within family-isolates among families. All factors were analyzed as fixed effects and the four-way interactions were used as the error term. All statistical analyses were conducted on maximum sporulation frequency data after arcsin transformation, and maximum sporulation capacity. The statistical analyses were conducted with SAS® (SAS Institute Inc. 1989).

To investigate the pattern of variation for each type of isolate, the sum of squares due to isolates was partitioned into effects of family and location isolates, each with three degrees of freedom.

Results

Gall Type

Gall type was statistically significant for all variables of sporulation ($P>0.05$) (Table 4-1), indicating that regardless of spore type, the frequency of sporulating trees

Table 4-1. Significance probability values of variance describing sporulation frequency and sporulation capacity of slash pine families after artificial inoculations with isolates of *Cronartium quercuum* f. sp. *fusiforme*.

		Probability>F [u]			
		Sporulation frequency[v]		**Sporulation capacity[w]**	
Sources of variation	**DF[x]**	**Spermatia**	**Aecia**	**ACY_{max}**	**SPY_{max}**
Isolates (Iso)[y]	15	0.40	**0.0007**	0.38	0.15
Family (Fam) Isolate	3	0.26	**0.04**	0.15	0.78
Location (Loc) Isolates	3	0.21	**0.05**	0.83	0.33
Family Isolate x Loc Isolate	9	0.48	**0.0008**	0.29	0.06
Gall Type[z]	1	**0.0001**	**0.0001**	**0.03**	**0.003**
Isolate x Gall Type	15	0.21	**0.02**	0.58	0.19
Fam iso x Gall Type	3	0.63	0.56	0.88	0.69
Loc iso x Gall Type	3	0.84	**0.01**	0.11	0.45
Loc iso x Fam. isox Gall Type	9	0.21	**0.03**	0.82	0.31
Family	3	0.07	**0.0001**	**0.02**	**0.03**
Family x Isolates	45	0.32	**0.0034**	0.21	0.19
Family x Family Isolates	9	0.15	0.09	0.94	0.87
Family x Loc iso	9	0.46	**0.005**	0.18	0.16
Fam x Family iso x Loc iso.	27	0.31	**0.004**	0.08	0.14
Fam x Gall Type	3	0.21	0.07	0.53	0.21
Fam x Fam iso x Gall Type	6	0.18	**0.02**	0.78	**0.03**
Fam x Loc iso x Gall Type	6	0.76	0.08	0.91	0.22
Error	12				

[u] The significance probability values associated with F tests; values in bold are significant at the 5% level.

[v] Sporulation frequency is the percentage of sporulating trees with a spore type:
Spermatia = arcsine of the square root of the percentage of seedlings with spermatia; and
Aecia = arcsine of the square root of the percentage of seedlings with aecia.

[w] Sporulation capacity is the proportion of gall surface area covered by a spore type:
ACY_{max} = maximum proportion of galled area covered with aeciospores; and
SPY_{max} = maximum proportion of galled area covered with spermatial exudates.

[x] Degrees of freedom

[y] Isolates are family- and location-isolates. Family-isolates are collections from a single gall collected from a specific family: A is an isolate collected from family R1; B is an isolate collected from family R3; C is an isolate collected from family R4; D is an isolate collected from family S2. Location-isolates are collections from locations 13, 17, 19, and 20 in Florida and Georgia.

[z] Gall types are: PSG = percentages of seedlings with galls smaller or equal to 25 mm long; and
PLG = percentages of seedlings with galls greater than 25 mm long.

with short galls (SG) were always lower than the frequency of sporulating trees with large galls (LG), 13 % and 62%, respectively (Table 4-2).

Sporulation Frequency

Overall, the frequency of spermatial (Sp) sporulation was greater (72%) than (Ac) aecial (4%) sporulation (Table 4-2). Although no family significant differences were found for spermatial frequency, a significant family effect was observed for aecial frequency ($P \leq 0.05$) (Table 4-1). The relative resistance of families can be observed in Table 4-3. For example, for aecial frequency, families separated into three groups, family R4 sporulated the most (27.11%) followed by families S2 (18.21%) and R3 (13.7%), and R1 (8.9%).

Considering spermatial frequency, families could be statistically separated into two groups; one with a high spermatial frequency (74.2%) represented by family R3, and the other with a lower spermatial frequency (average 60.4%), represented by families R1, R4, and S2 (Table 4-3).

The data reported here showed a significant geographical effect for aecial frequency, but not for spermatial frequency (Table 4-1). Galls produced by inoculum from location 20 sporulated more frequently than those induced by inocula from other locations, and galls produced by inoculum from location 19 sporulated the least (Table 4-4). Moreover, significant effects for the interactions were found only for aecial frequency (Table 4-1). However, because some family-isolates were associated with aecial sporulation and others were associated with non sporulation, it is likely that family-isolates were the main factor contributing to these interactions.

Table 4-2. Frequency of sporulation of fusiform rust galls on 1-year-old open pollinated slash pine progenies in the greenhouse after inoculation with *Cronartium quercuum* f. sp. *fusiforme*.

Sporulation[x] pattern	Living galls No.	Living galls (%)	Sporulation. type	Living galls No.	Living galls (%)	Sporulation type	Gall type[z]	No.	(%)	Sporulation type	No.	(%)
Sporulation	350	(75.3)	Sp	331	(72)	Spor.	LG	288	(62)	Sp	273	(59)
			Ac	19	(4)					Ac	78	(17)
			both	65	(14)					both	14	(14)
							SG	62	(13)	Sp	58	(12)
										Ac	6	(1)
							Total	350		both	2	(0.4)
None	115	(24)				None	LG	24	(5)			
Total	465						SG	91	(20)			

[x] Represents the frequency of trees that sporulated:
sporulation = the percentage of trees that had at least one spore type; and
none = the percentage of trees that did not had any spore type, the same as nonsporulating galls.

[y] The percentage of a given spore type:
Sp = frequency of spermatial sporulation; and
Ac = frequency of aecial sporulation.

[z] The gall type:
LG = large galls, galls are greater than 25 mm long; and
SG = short galls, galls are smaller or equal to 25 mm long.

Table 4-3. Sporulation frequency and sporulation capacity (maximum area of gall covered with spermatia (Spy_{max}) and aecia (Acy_{max})) in four families of slash pine after inoculation with *Cronartium quercuum* f.sp. *fusiforme*.

Sporulation Type	Families [w]			
	R1	R3	R4	S2
A) Sporulation frequency [x]				
Spermatia (Sp)	60.1 ab [y]	74.2 a	58.8 b	62.4 b
Aecia (Ac)	8.9 c	13.7 c	27.1 a	18.2 b
Sporulation	69.7 a	75.3 a	66.8 a	69.2 a
B) Sporulation capacity [z]				
Spy_{max}	2.1 a	1.7 ab	1.1 b	1.4 ab
Acy_{max}	3.9 b	6.5 b	13.7 a	9.10 ab

[w] R1, R2, R3, and R4 represent resistant families; S2 represents a susceptible family.

[x] Sporulation frequency is the percentage trees with a spore type:
Spermatia = percentage of trees with spermatial sporulation;
Aecia = percentage of trees with aecial sporulation.; and
Sporulation = the percentage of trees that had at least one spore type.

[y] Values within row followed by the same uppercase letter do not differ significantly according to Duncan's multiple range test (P=0.05).

[z] Sporulation capacity is the proportion of gall surface area covered by a given spore type:
Acy_{max} = maximum proportion of galled area covered with aecia;
Spy_{max} = maximum proportion of galled area covered with spermatia.

Table 4-4. Sporulation frequency of *Cronartium quercuum* f. sp. *fusiforme* on slash pine in four location-isolates collected in Florida and Georgia.

Location-isolates[y]	Sporulation frequency[x] (%) Spermatia		Aecia	
13	60.60	A[z]	18.6	B
17	62.34	A	15.80	B
19	67.70	A	8.50	C
20	71.00	A	25.00	A

[x] Sporulation frequency is the percentage of trees with a spore type:
Spermatia = percentage of trees with spermatial sporulation; and
Aecia = percentage of trees with aecial sporulation.

[y] Location-isolates are collections from locations 13, 17, 19, and 20 in Florida and Georgia.

[z] Values within a column followed by the same uppercase letter do not differ significantly according to Duncan's multiple range test (P=0.05).

Sporulation Capacity

Generally, significant differences for the proportion of galled covered with a spore type were found only between gall types and among families (Table 4-4), indicating that gall types and families differ in the amount of spores produced per gall surface area. However, for maximal spermatial capacity (Spy_{max}) (Table 4-3), no differences in families were observed, whereas family R4 had the maximum aecial capacity (Acy_{max}). Further, an interaction of family with family-isolate with gall type was observed for spermatial capacity (Table 4-1). However, for aecial capacity no significant effect of the interactions was observed.

Table 4-5. Sporulation potential of open pollinated, slash pine progenies after inoculation with 16 isolates of *Cronartium quercuum* f. sp. *fusiforme* at the Resistance Screening Center.

	Sporulation Potential[x] (cm^2)		
Families[z]	Spermatia	Aecia	Total
R1	0.42	1.00	1.42
R3	0.18	0.68	0.87
R4	0.16	2.45	2.61
S2	0.46	3.64	4.10
Mean	0.31	1.94	2.30

[x] Sporulation potential is the sporulated area of a given family calculated as

$SP_i = P_{LG} * S_{LG}*A_{LG}*I_{LG} + P_{SG} * S_{SG}*A_{SG}*I_{SG}$, where

SP_i is the sporulation potential of i^{th} family expressed in cm^2 per tree;

P_{LG} and P_{SG} are the proportions of trees with large galls and short galls, respectively;

S_{LG} and S_{SGc} are the proportions of sporulating trees with large galls and short galls, respectively;

A_{LG} and A_{SG} are the surface areas of large and short galls, respectively; and

I_{LG} and I_{SG} are the sporulation capacities expressed as the maximum proportion of a gall surface area covered by a spore type on large and short galls, respectively.

[z] R1, R3, and R4 are resistant families; S2 is a susceptible family.

Sporulating Potential

The sporulation potential was defined earlier as the surface area of a gall that produced a spore type. Generally, there was no indication that families with a high sporulation potential for spermatia have a high sporulation potential for aecia (Table 4-5). However, the susceptible family (S2) had the highest sporulation potential for both spermatia and aecia. The pooled sporulation potentials for spermatia and aecia ranged from 0.87 cm^2 for family R2 to 4.10 cm^2 for family S2. Families with lower sporulation potential than the overall mean may possess more resistance, in terms of gall areas that sporulate. Thus, considering the overall sporulation potential means, three types of response were observed: one in which family responses were below the mean average (families R1 and R3), an intermediate response (family R4), and a response in which families were above the average response (family S2).

Combining the information about sporulation frequency, capacity, and potential with gall type, it was observed that the tendency of a family to form large galls (susceptible symptoms) did not always correlate with the frequency of families with spermatial and/or aecial sporulation. For example, family S2 had the highest frequency of seedlings with large galls (89%) (Figure 4-1A), but had a lower sporulation frequency than that of family R1, which had 47% of large galls (Figure 4-1B), and lower number of spores per square centimeter of gall than family R4 (Figure 4-1C).

Discussion

Attention has been placed on resistance to fusiform rust with particular emphasis on phenotypic responses as potential indicators of plant defense mechanisms of resistance. In greenhouse tests at the Resistance Screening Center, gall types are used in an index to predict field resistance of slash pine families to fusiform rust (Hubbard 1981; Walkinshaw et al.1980). The index has greatly increased the correlation of greenhouse tests and field progeny tests with comparison with results using only presence or absence of galls. Trees with different mechanisms of resistance should be considered to maintain genetic diversity, thereby minimizing selection pressure on the pathogen, in the field.

The relative resistance (perhaps quantitative) of families is indicated by the percentage of galls which did not sporulated, sporulated in lower frequency, or had a lesser proportion of gall surface area covered with one or more spore forms.

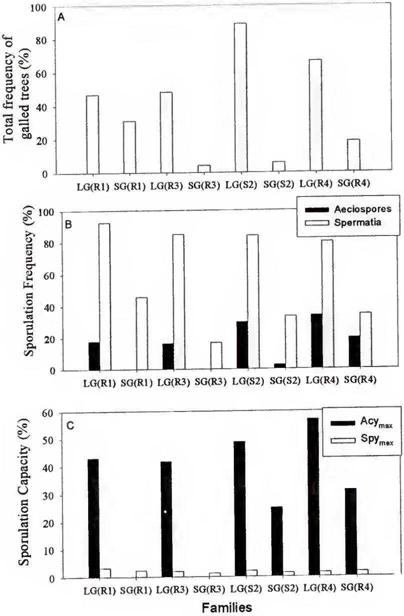

Figure 4-1. Total frequency of galled trees, sporulation frequencies and sporulation capacity of *Cronartium quercuum f. sp. fusiforme* on open-pollinated slash pine progenies expressing short (SG) and large (LG) galls. (A) Total frequency of galled trees was estimated based on the data of the single gall isoaltes (Chapter 3), which was used to obtain the experimental units for this study; (B) Sporulation frequency is the percentage of trees with spermatial droplets (spermatial frequency) and with aeciospores (aecial frequency); (C) Sporulation capacity (commonly refers to severity in terms of disease) is the proportion of gall surface area covered by a given spore type. Families R1, R3, and R4 are resistant families, and S2 is a susceptible family.

Gall Type

Because of the detailed observation and measurements on sporulation assessment in this study and in the previous chapter, it was possible to assess qualitative and quantitative sporulation responses. Assessments clearly indicated differences in sporulation pattern between large and short galls. The frequency of large galls that sporulated was five times greater than that of short galls.

Schmidt and Miller (1999a) raised the possibility of short galls being an indicator of partial resistance. Partial resistance has been associated with a reduced rate of epidemic development, reduced spore production, smaller lesion sizes, longer latent period, and a short infectious period (Parlevliet 1979). Partial resistance has been demonstrated commonly with cereal rusts, but partial resistance is also found in perennial pathosystems, such as coffee leaf rust (Eskes 1983). This effect was evident in this study. Short galls generally sporulated less frequently and had a smaller proportion of the gall area covered with spores than did large galls.

It was demonstrated in the previous chapter that frequency of short galls is an important contributing factor in predicting field performance to fusiform rust. Also, earlier studies have suggested short galls as indicative of resistance (Jewell and Snow 1972; Kuhlman and Powers 1988; Miller et al. 1976). These earlier studies provide strong evidence that seedlings producing short galls may be expressing partial resistance. Careful examination of families with a higher frequency of short galls could improve resistance to different pathotypes of *C. quercuum f. sp. fusiforme*, and provide a better understanding of the resistance mechanisms in this pathosystem.

Sporulation Pattern

In this study the overall spermatial sporulation frequency (71%) was much higher than aecial sporulation frequency (4%). Similar results have been reported by Kuhlman and Mathews (1985). In contrast, Kuhlman (1981) and Chappelka and Schmidt (1984) working with mixed pathogen populations found the greatest proportions of galls forming aeciospores in field studies. Here sporulation patterns have been associated with host and pathogen variability, inoculum concentration, and environmental conditions. Schmidt and Miller (1999b), examining the effect of inoculum concentration on spermatia production, found a direct relationship of high inoculum concentration and aeciospore formation, and an inverse relationship between inoculum concentration with spermatia. Because of the high inoculum concentration used in the present study (20,000 spores/ml), one might expect greater aecial sporulation. However, a major difference in this study was that single-gall isolates were used, whereas in other studies mixed inoculum sources were used, and Schmidt and Miller (1999b) only looked at large galls.

Spermatia and aeciospores formed more frequently on seedlings of the susceptible family (S2) than on seedlings of the three resistant families. It was observed that the relative resistance of families significantly influenced the sporulation pattern. However, when considering only resistant families, no clear pattern was found. For example, family R4 produced significantly more aeciospores than spermatia, whereas family R1 produced more spermatia than aeciospores (Table 4-3).

The following hypotheses are proposed to explain the high frequency of spermatia and the low frequency of aeciospores. First, pathogenic variability in slash pine families inoculated with single-gall isolates may not be as variable as previously reported (Dinus et al. 1975; Snow et al. 1975; Snow et al. 1977), or that pathogenic variability does not equate to mating types. If most of a gall is colonized by one mating type, dicaryotization or aecial formation could be limited. Recently, Hamelin et al. (1993) demonstrated that unspermatized spermogonia of white pine blister rust consist of a single genotype. Likewise, Kasanen (1998) found similar homogeneity within cankers of *Peridermium pini*. Thus, it is possible that the same homogeneity may be found with single-gall isolates of *C. quercuum f. sp. fusiforme*. Second, one pathogen genotype may be more fit and completely colonize a gall more rapidly than other genotypes and limit aeciospore formation. Third, although spermatia of *Cronartium* spp. may be functional, their ability to spread and cross-fertilize could be partly due to a lack of suitable insect transfer or favorable environmental conditions. However, these latter explanations seem unlikely as they would have affected all families.

From a practical epidemiological standpoint, the families with limited sporulation, especially those with less aecial sporulation, could contribute to decreased infection of oak in the field, therefore minimizing the frequency of pathotypes capable of attacking genetically improved trees.

Conclusions

Families characterized by short galls had reduced frequency of trees with sporulating galls forming spermatia or aeciospores and fewer numbers of spores per gall than families with large galls. Clearly, these characteristics of families with short galls are components of partial resistance. Therefore, it is suggested that families with high frequencies of short galls have a resistance similar to that of partial resistance found in leaf rusts of cereals and coffee leaf rust. Although the resistance of families significantly influenced the sporulation pattern, spermatia, as well as aeciospores, generally formed more on the susceptible family (S2) than on the resistant families. However, when considering only resistant families, no clear pattern was found between the two spore types. For example, in comparison to other families family R4 showed higher frequencies of aecial sporulation, whereas family R1 showed higher frequencies of spermatial exudates. This suggests that, although some families form spermatia in lower frequencies, they will not necessarily have a low frequency of aecial sporulation. Therefore, both spore types should be considered in evaluating partial resistance.

The differential response of families for spermatial and aecial formation might be an indication that single-gall isolates do not have as much pathogenic variability (at least mating type variability) as previously suggested. It is also possible that these responses occurred because families may have different types of resistance.

From a practical epidemiological standpoint, the responses of seedlings with limited sporulation, especially those with less aecial sporulation, would contribute to limited infection of oak in the field, therefore minimizing the frequency of pathotypes capable of attacking genetically resistant pines.

CHAPTER 5

MORPHOLOGICAL ASPECTS OF HOST-PARASITE INTERACTION: SOME OBSERVATIONS ON THE RESPONSES OF SLASH PINE (PINUS ELLIOTTII VAR. ELLIOTTII) SEEDLINGS TO *CRONARTIUM QUERCUUM F. SP. FUSIFORME*

Introduction

Cronartium quercuum (Berk.) Miyabe ex. Shirai f. sp. *fusiforme*, the causal agent of the fusiform rust disease, is an obligate parasite that possesses five spore states. The primary hosts are oaks (*Quercus* spp.) of the red oak group, and the secondary hosts are several southern pines, particularly slash pine (*Pinus elliottii* var. *elliottii* Engelm) and loblolly pine (*Pinus taeda L.*) (Czabator 1971; Dwinell 1977). Selection and breeding for resistance have substantially reduced fusiform rust incidence (Powers et al. 1993; Pye et al. 1997; Schmidt et al. 1981). Evidence for variation in resistance among pine families to the pathogen is based on phenotypic symptoms expressed on seedlings 6-12 months after inoculation. Symptoms typically evaluated are the presence, shape, and size of galls, and small stem lesions that do not produce swellings (SYMNOS) (Hubbard 1981; Walkinshaw et al. 1980). In 1958, Jackson and Parker (1958) described in detail the internal anatomy of the typical fusiform rust gall on loblolly pines. The tracheids of the colonized xylem were irregular in shape and shorter than the tracheids in healthy tissue. The disease-induced rays contained more cells and were greatly enlarged. Phloem parenchyma cells increased in number and size. In a histological study of seedlings of slash pine, following inoculation with *C. quercuum* f. sp. *fusiforme*, Miller et al. (1976) found four distinct host-parasite reactions: (1) susceptible, with the rust gall anatomy typical of a susceptible pine-fusiform rust interaction; (2) incompatible, with the host showing no apparent symptoms due to the inability of germinated basidiospores to penetrate and infect the host; (3) subliminal infection, in which the host was infected but no morphological symptoms were observed, and sparse mycelium and a few scattered small haustoria were presented internally; and (4) hypersensitive-like reactions, resulting either from stem infections or from needle trace infections, in which the fungus is confined to reaction zones of necrotic cells in the stem. Macroscopically, these areas of necrotic cells are identifiable by either limited symptoms with no swelling (SYMNOS) or to galls less than 25 mm in length (short galls). In the short galls, the pathogen induces typical galls of a restricted size. In the SYMNOS reaction, there is no indication of pathogen viability in the affected host tissues, and no stem swelling.

The anatomical expressions of resistance to the pathogen in inoculated slash pine seedlings were termed "resistance zones" by Jewell et al. (1976). Reactions, including reaction zones and periderm formation in the fusiform-rust pathosystem, have been reported as evidence of possible mechanisms of host resistance (Jewell 1988; Jewell and Snow 1972; Jewell and Speirs 1976; Miller et al. 1976). According to Jewell et al. (1980a), the initial formation of these reactions is evident 30 days after inoculation and apparently is initiated in the primary tissue system of the host, with the primary meristems developing a limited but self-perpetuating abnormal parenchyma-like area of tissue in the host that contains the pathogen. These reactions prevent fungal spread or fungal growth within the host (Jewell et al. 1980a).

Initially, percentage of galled seedlings was used at the Resistance Screening Center to evaluate resistance to fusiform rust. Fusiform rust resistance in pine was a measure of lower frequency of seedlings that had galls compared with the frequency of seedlings that have galls in susceptible control families (Kuhlman and Matthews 1993). These galls varied in size, from a slight swelling (short galls) up to two to three times the normal stem diameter (galls were large and fusiform shape). They also varied in appearance; the gall surface was either smooth, the gall surface has the same texture as the unaffected stem tissue above and below the swelling, or the gall surface was rough, with reddish-brown necrotic and sunken areas (Miller et al. 1976; Powers 1971; Walkinshaw et al. 1980). Griggs et al. 982), studying the symptom types SYMNO (SY), short galls (SG) (< 25 mm long), and large galls (LG) (≥ 25 mm long), found that slash pine seedlings expressing SG resist colonization by the rust fungus, whereas seedlings with LG are extensively colonized by the fungus. Walkinshaw et al.(1980) and Hubbard (1981), using data from the Resistance Screening Center, evaluated four forms of indices for use in predicting relative field resistance. They showed that short galls, purplish discoloration on the stem and no swelling, smooth galls, and rough galls could be used to predict relative levels of rust resistance of pine families in the field. Since then, gall shape and appearance have been used as further indications of resistance and susceptibility to fusiform rust. Currently, the Resistance Screening Center uses an index developed by Walkinshaw et al. (1980) that classifies levels of rust resistance. This method allowed the correlation of greenhouse traits to resistance under field tests using the differential gall types and purplish spot on the stem and no swelling. This Index combines the four-symptom types and ranks the potential resistant families. The differential phenotypic response and the different anatomical and size features of symptoms of slash pine seedlings to *C. quercuum* f. sp. *fusiforme* suggest different mechanisms of resistance. Information from histological examination of phenotypic expressions of resistance will aid in the understanding of the process of pathogenesis involved at the tissue and cellular level, and could aid in the development, evaluation, and selection of resistance. The histopathological basis for the importance of these symptom types in predicting "field resistance," however has not been investigated, particularly relative to the genetic control of resistance in slash pine to *C. quercuum* f. sp. *fusiforme*. The objectives of this study were to compare, microscopically, host responses to *C. quercuum* f. sp. *fusiforme* and patterns of colonization in slash pine seedlings with the RO, SO and SG symptoms.

Material and Methods

Seeds of six open-pollinated slash pine progenies, obtained from seed collections for the Rust Virulence field tests, were used in this study. Their maternal parents were selected in the coastal plain of Florida and Georgia. The parents were chosen from a population previously selected based on their breeding values for rust resistance (R50) and volume growth from field progeny tests. An R50 expresses the expected percentage of offspring from a given parent that will be infected with fusiform rust at a field site, at which unimproved material expresses 50% disease. The qualitative ratings of rust incidence, the general location of the maternal parents, the R50 and volume ratings from the Cooperative Forest Genetics Research Program, at the School of Forest Resources and Conservation at the University of Florida, are given in Table 2-2a and Table 2-2b in Chapter 2. Based on these ratings, families R1 and R4 were rated as resistant; families R2 and R3 as very resistant; and families S1 and S2 as very susceptible (Schmidt unpublished data). The performance ratings of these families in four field plantings (13, 17, 19, and 20) in the Rust Virulence study appears in Table 2-1a and 2-1b in Chapter 2.

Six months after inoculation, at the Resistance Screening Center (RSC), seedlings from each family (inoculated in Chapter 2) were collected with the following gall symptoms: rough galls (RO), smooth galls (SO), and short galls (SG). Inoculation procedures, using a mixture of isolates from the susceptible families (S1 + S2), are described in Chapter 2. The galls on each seedling were cut from the stems and subdivided into segments prior to fixation in a 18: 1: 1 solution of formalin-propionic acid-ethyl alcohol (50%) (FPA). The segments were taken from different positions on the galls: uppermost region, region 1, region 2, region 3, and lowermost region (Figure 5-1). In the case of RO, samples were taken from both the region of maximum enlargement and the constricted area, which characterizes this gall type.

After fixing, gall segments were dehydrated through a tertiary butyl alcohol series (50 to 100%), and embedded in Tissuemat® (melting point 56.5 °C) (Fisher Scientific Company Pittsburg, PA). Embedded tissues were sectioned (5- to 12-μ-thick sections) using a rotary microtome after fixation to glass microscopic slides using Haupt's adhesive. Sections were dewaxed in three changes of 100% xylene, passed through a series of xylene and absolute ethyl alcohol (ETOH) (1:1), absolute ETOH, and 70% ETOH. Sections were stained with Pianeze's IIIB (Vaungh, 1914), and eight sections from each individual gall segment were observed under a light microscope for host reactions and fungal colonization pattern. Photomicrographs of representative sections were taken using either an Olympus/BMH or a Nikon OPTIPHOT II system.

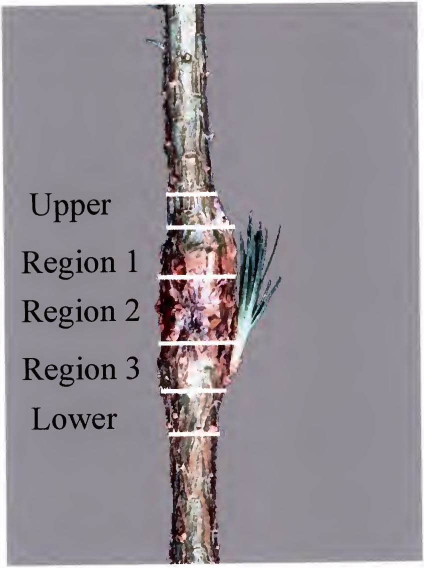

Figure 5-1. Nine-month-old slash pine with a rough gall. Areas of the gall are delineated and labeled for identification of histological specimens collected.

Results

Tissue samples taken from different portions of slash pine galls, morphologically designated as short galls (SG), smooth galls (SO) and rough galls (RO) were sectioned, stained and examined microscopically. Virtually, all symptom types developed areas of necrotic cells within the host tissues, herein referred to as reaction zones (Rz).

There were significant differences in the appearance, size and pattern of colonization among the reaction zones of the different gall types. Reaction zones were most evident in the portion of the galls with maximum swelling (Region 2) (Figure 5-1). Characteristic of this region was the presence of a zone of dark-pink-stained parenchyma cells formed along the cortical-phloem region, deep in the cortex, suggesting a quiescent reaction zone. Despite the similarities, discrete differences were found among the quiescent reaction zones of different galls. In SG no necrotic cells were observed in this zone, but cells stained much darker than the cells of RO; whereas in RO and SO, small reaction zones were observed. However, the necrotic areas in the SO were much smaller and more numerous than those in the RO. Another major difference in SO was the abundant distortion and proliferation of the inward portion of the reaction zone. Cells in the reaction zone stained green, and some portions of the reaction zone extended to the cambium. In most of the galls, no typical reaction zone was observed in the uppermost region.

Smooth Gall (SO)

Reaction zones, most abundant within region 2 of the gall, extended upward from region 2 to region 1 and downward from region 2 to the lower region of the gall. Most reaction zones formed in the innermost portion of the cortex extending toward the phloem region (Figure 5-2A). The major differences of the reaction zones described in the SO and the reaction zones described by Jewell et al. (1980b) and Miller et al. (1976) were the smaller size of the reaction zone here, the staining reaction of the cortical tissues (cells of the reaction zone stained green, indicating little tanninization), and the presence of apparently functional hyphae. The same differences were observed between the reaction zones of RO and SG. Here also, the fungus was limited to the reaction zone and did not reach the cortex region, which appeared healthy and normal. Hyphae, when present, appeared functional, based on the staining reaction (Figure 5-2C). The following host anatomical responses are described by region of the galls.

Region 1- The reaction zone in region 1 of the SO resembled those reaction zones of the uppermost region. A layer of cortical cells with intense pink staining was observed between the reaction zones (Figure 5-2B). This layer was formed by the same type of cells that bordered the reaction zone delimiting the outermost cells of the cortex region. Also, based on stain reaction, the reaction zone appeared intermediate between the reaction zone in RO and SG.

Region 2- A continuous reaction zone was formed deep in the cortex (Figure 5-2A). The reaction zone was characterized by a smooth surface, and mostly dark-green stained cells (Figure 5-2C). In some areas of the gall, cells of the periderm were formed by

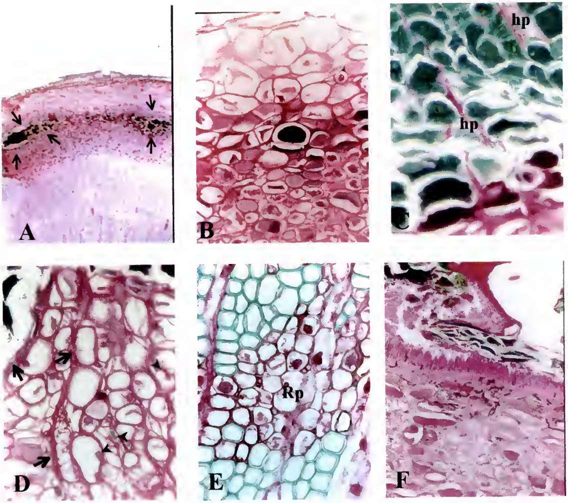

Figure 5-2. Histological observation through region 1 of a smooth gall (A-C): A) Cross-section showing reaction zone of necrotic cells interspersed with affected cells (x10) (arrows); B) Cortical cells between the reaction zones of necrotic cells; C) Portion of the reaction zone showing lighter-stained reaction and limits of colonization by the fungus (hp=fungal hyphae) (40x); D) Cross-section on region 2 of a smooth gall showing the affected region beneath the reaction zone of necrotic cells and abundant colonization. Note the thick hyphae (arrows) and apparent nonfunctional haustoria (arrowheads) (40x); E) Cross section of region 2 of a smooth gall showing the reaction parenchyma (Rp); and F) Cross section of region 2 of a smooth gall showing spermatium formation beneath the reaction zone (20x).

sclerenchyma cells. In the innermost portions of the reaction zone, hyphae were unusually multi-septate, larger, distorted, and often filled with granular-like material. The reaction parenchyma was observed at the lower portion (Figure 5-2E). Inward from the reaction zone, colonization was dense, and both hyphae and haustoria were abundant. In some instances, haustoria appeared nonfunctional as they stained green (Figure 5-2D). In the cambium, colonization was dense, and cells were filled with granular material that stained light.

Lower region- Reaction zones in the lower portion of the gall were nearly as continuous as that in the reaction zone of region 2. A major difference was that the cells in the reaction zone appeared to have more intercellular space with apparently functional mycelium, and the cells in the cortex had abundant abnormalities, were deformed,, and appeared to have proliferated. Spermatium formation was observed at one side of the gall beneath the reaction zone in the cortex (Figure 5-2F). Abnormalities and colonization were similar to those described by Jewell et al. (1962).

Short Galls (SG)

As reaction zones were very similar in all sampled sections of the SG. Therefore the differences among the areas are not presented by region of the gall.

Mostly, reaction zones formed as a single and continuous layer in the innermost portion of the cortex (Figure 5-3A). The reaction zones in SG were well developed, deep in the cortex, and wider (averaging about 7.41-9.88 μ wide) than the reaction zones of the other gall types. The cells of the reaction zones showed the typical anatomical abnormalities described for reaction zone in slash pine (Jewell 1988; Jewell and Speirs

1976), which included heavily tanninized host cells, some of which contained apparently nonfunctional haustoria; a periderm, or periderm-like cells, and darkly-staining parenchyma- like cells within and bordering the reaction zone. Host tissues adjacent to the reaction zones were distorted, mostly by hypertrophy, and not considered normal for pine. The samples also revealed some differences in or adjoining the reaction zones that were limited to samples from SG. The cells in the innermost portion of the reaction zones were not uniformly necrotic, and, judging by staining reactions, contained both functional (stained red) and nonfunctional (stained yellow-green) haustoria and hyphae (Figure 5-3B). Haustoria and hyphae were abundant in and near the phloem-ray cells, where hyphae grew radially across the cambium into phloem and xylem (Figure 5-3D). These observations were similar to the pseudoresistant reactions on progeny from (shortleaf x slash) x shortleaf pine crosses (Jewell 1988). However, in the present study the pathogen did not escape the reaction zone.

The pathogen was well established in parenchyma cells in both the cortex-phloem and the xylem of the host. Phloem cells were greatly modified, and an apparent increase in cell numbers increased within the width of the phloem and reduced the intercellular spaces. There appeared to be an increase in number of parenchymatous-cells filled with oil-like materials (Figure 5-3C). These cells also had unusual cell wall characteristics. At the widest portion of the reaction zone, the abnormal phloem extended inward to the cambium and the cambial cylinder bent inward toward the xylem region.

Reaction parenchyma was observed radially opposite to the reaction zone of the cortical-phloem, marking the innermost extent of the pathogen. This often occurs in a typical fusiform rust gall and indicates the host's initial response to the pathogen (Jewell et

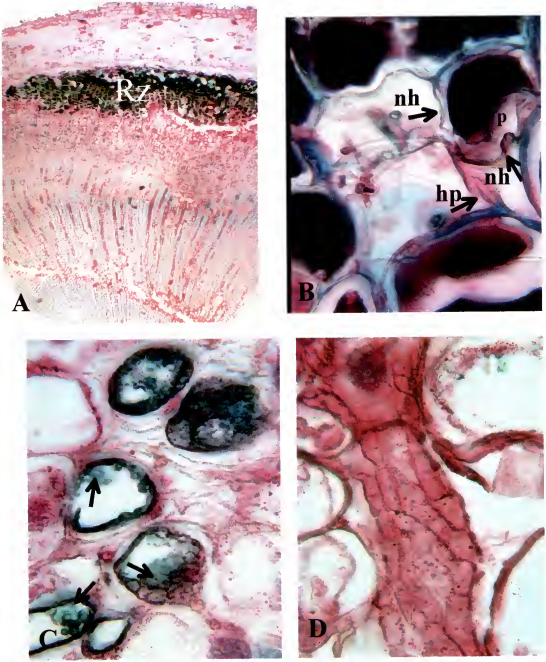

Figure 5-3. Histological observation through region 1 of a short gall with: A) Reaction zone (Rz) in the cortex. (10x); B) View of cortical cells in the reaction zone. Note large intercellular hyphae, apparently nonfunctional haustoria (nh) and hyphae (nhp), and functional hyphae (hp); C) Phloem cells filled with oil-like material (arrows) (40x); and D) Colonization inward from reaction zone, showing intense radial growth through ray cells. Note unusual thickness of the hyphae (x100).

al. 1962) (Figure 5-3A). Differences of the reaction parenchyma observed in SG and that described in a typical gall were related to the higher number of haustoria in the ray cells and thicker hyphae found in the typical galls (Figure 5- 3D). Occasionally, these parenchyma cells were also found in the outer xylem region close to the innermost region of the cambium.

Major differences in the reaction zones of SG were most evident in region 2 of gall. The reaction zones were deep in the cortex, approximately 30µ inward from the epidermal layer, than the reaction zones of SO and RO (Figure 5-4A). Also, the reaction zones were separated by a dark pink zone very similar to that in the upper part of the gall. Haustoria in the reaction zones were distorted or encrusted. A periderm of about two cells in width, formed by sclerenchyma or epidermis-like cells, had developed around the outer portion of the reaction zone and the adjacent layer (Figure 5-4B). Abnormalities and colonization in the outer portion of the reaction zone were very similar to those of the typical fusiform rust gall (Jewell et al. 1962); phloem cells were very disorganized and larger than normal.

Hyphae beneath the reaction zone appeared much thicker than in normal galls. However; few abnormalities were found in the tissue adjoining the reaction zone at the other side to that which extended from the pith to the epidermis. However, in both cases haustoria were considerably limited in number inward to the reaction zone (Figure 5-4C). The reaction zone in the lower region of the SG had the same characteristics as the reaction zone of the other gall regions, but was continuous along the axis of the stem. A major difference was the amount of distortion and colonization observed in the outer cortex region. In the affected cortical area, degeneration and disorganization of cortical

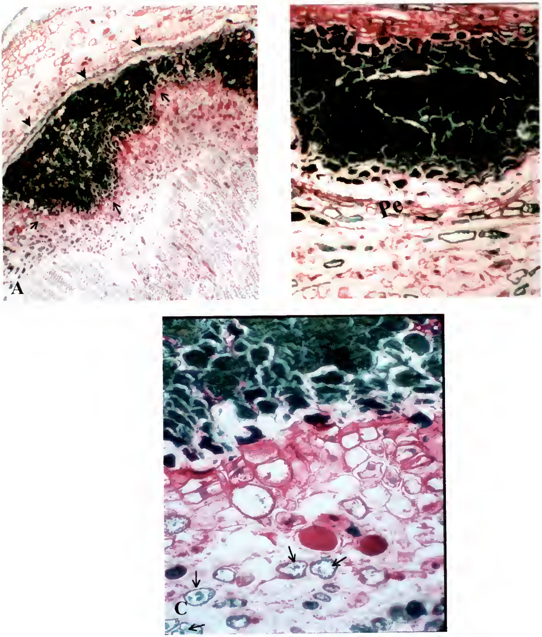

Figure 5-4. Histological observations of cross sections of region 2 of a short gall (SG) showing: A) wide reaction zone of necrotic cells and a layer of cells (arrowheads) between the reaction zone and non affected cells, and between the reaction zone and affected cells (arrow) (20x); B) view of an internal portion of the reaction zone layer of periderm (Pe); and C) the inner portion of the reaction zone showing scattered colonization and affected cortical and phloem cells with deposits of oil-like material (arrows).

cells altered their identity. Hyphae were thinner than in region 2 and abundant in the intercellular space; haustoria, which are typically abundant in fusiform rust galls, were few or absent.

In the xylem region, more cell proliferation and little colonization were observed in the reaction-parenchyma and in the xylem-ray cells. Perhaps, this indicates a quick host reaction, as ray cell proliferation contained sparse mycelia, which were more abundant at the proximity of the reaction zone.

Rough Galls (RO)

Generally, cells of reaction zones were more intensively stained, and showed more abnormalities than the cells of the reaction zones of the other gall types. Host abnormalities were evident and conspicuous in regions 1, 2, and the lower portion of the gall. Therefore, the host anatomical responses are described by region of the gall. The internal anatomical structure of region 2 was examined by histological sections from both the constricted areas of region 2 and the zone of maximum diameter.

Region 1- Reaction zones were interconnected by symptomatic tissues in the inner cells of the cortex beneath the point of infection, approximately 25µ inward from the epidermis (Figure 5-5A, B, C). A second reaction zone area formed just inward from the epidermis and was partially sloughed off (Figure 5-5D). Necrotic cells of reaction zones stained dark-green; cells between the reaction zone stained dark-pink. The largest reaction zone averaged about 9.88 x 1.48 µ, while the smallest averaged about 1.48 x 0.75 µ. The symptomatic cells between the reaction zone resembled typical gall tissue as previously reported and illustrated by Miller et al. (1976), Jewell et al. (1980a) and Jewell (1988).

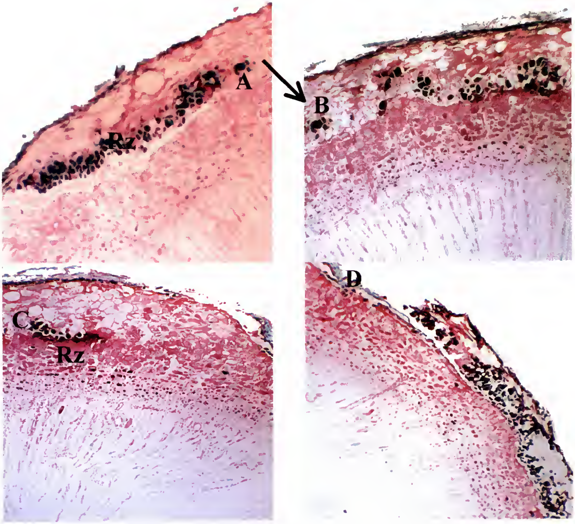

Figure 5-5. Four transverse sections through the region of a rough gall (RO) showing reaction zones (Rz) of different sizes. Note reaction zones that started at the epidermis (A) and spread in the inner cortex (B) and then, together with few abnormalities arising in the inner cortex and phloem (B to C), has partially isolated the outer cortex external to it. Following this area, another reaction zone has become severely necrotic (from C to D), appears to be dying, and eventually is sloughed off.

The difference was the colonization of the cortical tissue associated with the tissues from gall area without depressions. Most of the cells in the reaction zones stained dark-green but contained identifiable pinkish/blue cortical cells that were severely disorganized. Cells between reaction zones were not as disorganized, and stained dark-pink. Hyphae and haustoria in the reaction zones were largely encrusted and collapsed. A few scattered intercellular hyphae were observed that, based on the staining reaction appeared dead. The fungus was effectively restricted to the reaction zone. The layer of cells inward and adjacent to the reaction zone was swollen, with few distortions and little proliferation. Generally, more hyphae than haustoria were observed. The quantity of both hyphae and haustoria increased from the margins of the reaction zone to the cambium. Dense colonization was present in the innermost cells of the cortex beneath the reaction zone, in the phloem, and along the cambium. Cortical and phloem cells were very disorganized, mainly in the phloem region inward to the reaction zone, where cells were also crushed. In this region hyphae were swollen and haustoria were encrusted. In contrast, hyphae and haustoria appeared normal in the region radially opposite to the gaps inward from the phloem.

Ray cells in the gall tissue were more numerous and distorted compared to normal ray cells. They averaged 1.18-2.36μ wide and 11.8 μ high (about eight cells in height). Normal rays averaged three cells in height and two cells wide (Jewell et al. 1962). Hyphae, observed mainly in ray tissue, radiated towards the cambium, phloem, and xylem; however, they were much thicker than the hyphae in other cells. The intercellular hyphae were thin and relatively sparse with very few haustoria. Often cells beneath the reaction zone were distorted and larger than normal. Some cells in the cambium toward the xylem were disorganized and filled with granular material, which stained pink. Haustoria and hyphae were restricted to the ray cells.

Region 2-The portion of the gall with the maximum stem swelling, adjacent to the constricted areas, revealed anatomical abnormalities characteristic of typical fusiform rust galls in slash pine, as described by Jewell et al. (1962). However, there were some isolated reaction zones in the cortical region which were located inward from the epidermis toward the innermost of the cortical region and extended longitudinally through the sample (Figure 5-6A). Differences in the reaction zones of region 2 as compared to in region 1 included formation of a discrete layer, more intensely stained and not as deep into the cortex. Intensive colonization extended from the innermost part of the reaction zone toward the phloem and cambium. The colonization and fungal growth in this area was restricted to the innermost portion of the superficial reaction zone. Overall, host abnormalities were very similar to that of a typical fusiform rust gall; hypertrophy, hyperplasia of the cortex, phloem and proliferated phloem-ray cells and shorter pine tracheids were common (Jewell et al. 1962). A major difference in these galls was the colonization of the fungus, with abundant hyphae but scattered haustoria observed along the cortex.

Transversal sections of the host cells in the constricted area of region 2 revealed an isolated reaction zones restricted to the cortex region (Figure 5-6A,B). Major differences in these reaction zones were that cells were larger in diameter, had no distortion or proliferation, were more intensively stained, and more necrotic than cells in the previous reaction zone (Figure 5-6B). The pattern of colonization in the inner portion of the reaction zone was similar to that previously reported and illustrated by Jewell et al. (1962)

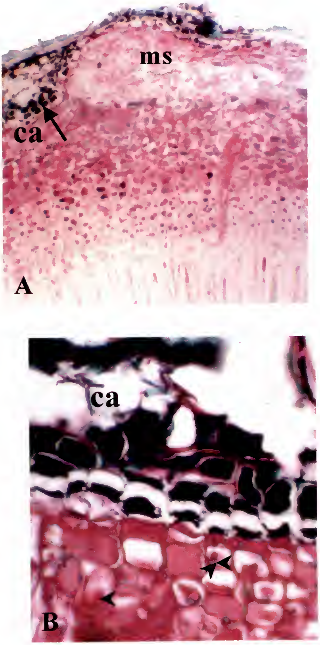

Figure 5-6. Histological observations through region 2 of a rough gall. A) Zone of maximum swelling (ms) and constricted or depressed area (ca). A discrete reaction zone is present in the depressed portion of the gall; B) Cross section of the reaction zone in the constricted area (ca) of the gall. Note the intensively stained necrotic cortical cells, and limited colonization with haustoria (arrowheads).

and Miller et al.(1976). Disorganization, distortion and hyperplasia of cells beneath reaction zones were very common. Also, the walls of xylem cells in the inner portion were thicker than cells of normal xylem.

Tapered lower regions- A reaction zone with the same characteristics as in the constricted portion of region 2 was present in the cortex on one side of a gall. Major differences from reaction zones in region 2 were the accumulation of granular material along the cortical-phloem region. Also, in the xylem region, inward the reaction zone, tracheids were unusually distorted with abundant haustoria. On the other side of the gall, where the reaction zones were not found, the fungus was present in the cortex and the radial progress of the fungus had advanced to the phloem tissue and the cambium region. However, little proliferation of ray tissue and almost no hyperplasia and hypertrophy was observed.

Discussion

Although artificial inoculation in the greenhouse was used to control the potential causal factors of the family x year interaction present in the field trials, variability was still present in the greenhouse data. The inocula used may approach the wild type (six galls each from the two susceptible families), and thus, represent the variability in inoculum in the surrounding area. Another source of variability was introduced with the seedlings selected for inoculation. They were open-pollinated progenies of slash pine. The variability in open-pollinated families and the diversity within the fungal isolates could account for some of the variability in the data, and probably contributed to results. The problem of limiting heterogeneity in the host and in *C. quercuum* f. sp. *fusiforme* has long been challenging and can best be accomplished with single spore fungal cultures and clonal host material. However, single spore isolates are extremely difficult to obtain due to the apparent synergism among basidiospores (Amerson et al. 1985)

While some of the findings confirm the results of previous histological studies (Jewell et al. 1962; Jewell et al. 1980a; Miller et al. 1976), the principal objectives were to investigate the host response (galls) that have been given the designation of small galls (SG), where the pathogen induces typical galls of a restricted size in the host (< 25mm); smooth galls (SO), where the gall surface is smooth, and rough galls (RO), where the gall surfaces are rough and reddish-brown with obvious necrotic sunken areas (Jewell 1988; Jewell et al. 1980; Miller et al. 1976). These symptom types, occurring on inoculated seedlings, have been determined to be of value in predicting the relative resistance of selected families of slash pine to fusiform rust when planted in the field (Hubbard 1981; Walkinshaw et al. 1980). The basic differences and similarities detected in the different gall types are briefly summarized in the Table 5-1.

Anatomical resistance expressed as reaction zones in seedlings of certain slash pine families is a well-documented response to infection by *C. quercuum* f. sp. *fusiforme* (Jewell et al. 1980a; Lundquist and Miller 1984; Miller et al. 1976). It is thought to be initiated in the primary stem tissues and in adjoining infected primary needle traces. The response is more obvious in the outer cortex, where the reaction zones stained intensively, possessed living or nonliving hyphae and haustoria, had cell wall thickenings, possessed cell content dissolutions (Jewell et al. 1980b), and sometimes had a distinctive granular appearance (Miller and Cowling 1977). The presence of the fungus in the outer cortex

Table 5-1. Morphological and anatomical characteristics of slash pine families expressing resistant host responses (galls) to *Cronartium quercuum* f.sp. *fusiforme*

Characteristics	Smooth galls	Short galls	Rough galls
Reaction zone in the cortex of the gall	Present	Present	Present
Position of the reaction zones in the cortex	Shallow depth in the cortex. Interconnected with symptomatic tissue	Very deep in the cortex	Closer to epidermal layer
Reaction zone pattern	Small and narrow, with apparently functional mycelial often observed	Wide and continuous in the circumference of the stem functional and nonfunctional mycelia in the reaction zone	Small and numerous, interconnected with symptomatic tissue typical of a fusiform gall, with no hyphae or haustoria within the reaction zone.
Staining of cells in reaction zone	Green, indicating little tanninization	Dark green, indicating some tanninization	Very dark green (almost black), indicating tanninization
Periderm	A layer of periderm bordering the innermost portions of the reaction zone	Well-developed periderm bordering the outermost and innermost portions of the reaction zone	Absent
Localization of the fungus	Limited to the innermost portions of the reaction zone	Limited to the innermost portions of the reaction zone	Limited to the innermost portions of the reaction zone
Hyphae and haustoria	Present but limited	Present but limited	Present but limited

cells is associated with dark pigmentation, likely caused by stimulation of phenolic compounds, including phenols and tannins (Lundquist and Miller 1984; Rowan 1970).

In the present study, all observed symptoms developed reaction zones similar to those found in earlier studies (Jewell et al. 1980a; Jewell et al. 1980b; Miller et al. 1976; Miller and Cowling 1977). While the resistant symptoms had many anatomical characteristics in common, there was some variability, and individual symptoms showed unique characteristics. The most common feature observed in resistant symptoms was the formation of reaction zones in the cortex regions. However, the size, rate of development, and staining reaction or amount of pigmentation in the reaction zones were the conspicuous histological differences among the three symptom types (Figure 5-7A,B,C).

In the RO, reaction zones were not formed as a continuous layer of necrotic cells in the cortex. The innermost regions of the reaction zones sometimes were bordered by a thin layer of sclerenchyma cells resembling a periderm. Often these areas were associated to the constricted areas on the stem. The cortical area between the reaction zones possessed the same characteristics as that in a typical fusiform gall and corresponded to the swollen regions in the stem. Compared with the reaction zones in the SG, the reaction zones in the RO were smaller, more intensively stained, and hyphae and haustoria were rare and, when present, appeared dead.

The reaction zones in the SG occurred deep in the cortex and formed a continuous ring around the stem circumference. They were well formed, much wider, less intensively stained, and surrounded by a more well-defined periderm, when compared to the reaction zones in the RO, and occasionally both functional and nonfunctional haustoria were observed in the reaction zone (Figure 5-7C). Major differences between RO and SG

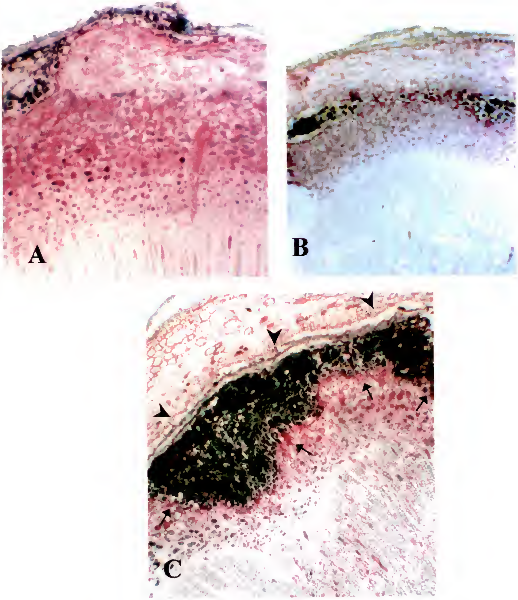

Figure 5-7. Summary showing a typical reaction zone of: A) rough gall; B) smooth gall; and C) short gall. Micrographs are sections from the region 2 of a gall.

included were the presence of a well-defined periderm, presence of granular materials, disruption of the cambium, and a much more conspicuous reaction zone in SG.

The extent of response and pathogen development in and adjoining the reaction zones of RO support the hypothesis that families expressing this symptom-type have lower level resistance than families expressing SG. The reaction zones in the constricted areas were often associated with bark lesions, resembling those of SYMNOS, while the swollen areas of the stem had characteristics similar to that of a typical fusiform rust gall.

Following are some possible explanations for the histological expressions in slash pine families developing RO. One possibility is the presence of a resistance type associated with gene dosage. For example, the infection types of homozygous (double-gene dosage) for resistance would condition SG, the heterozygous (single-gene dosage) for resistance would condition RO, and homozygous for susceptibility would condition large galls. A similar approach has been used to explain partial resistance in the cereal-rust pathosystem in the presence of major genes (Parlevliet 1987). Also, the frequencies of rust biotypes incompatible to the slash pine families tested may have been present at low and apparently stable frequencies, but high enough to trigger a more pronounced compartmentalization reaction at the infection sites. On the other hand, areas infected by a compatible mycelium and those adjacent to them may produce galls more readily than others following infection. This reaction also may suggest the presence of two resistance systems in the pathosystem, a multigenic system and a single gene system. Recently Wilcox et al. (1996) identified a major gene for fusiform rust resistance in loblolly pine. Morever, Kinloch (1982), Miller et al. (1976), and Walkinshaw (1978) suggested that resistance to *C. quercuum* f. sp.

fusiforme in slash pine families expressing different gall-types appears to operate in a nonspecific manner.

Resistance to *C. quercuum* f. sp. *fusiforme* in slash pine families expressing SG appears to operate in a nonspecific manner, as suggested by others (Kinloch 1982; Miller et al. 1976; Walkinshaw 1978). A major anatomical response of families expressing this trait was the production of a sequential periderm surrounding the reaction zones. The presence of hyphae and haustoria in the interior cells of the reaction zones indicated that these structural barriers were often not effective at preventing spread of the pathogen. The presence of periderms also has been demonstrated in other rust-affected trees (Jackson and Parker 1958; Jewell and Speirs 1976; Miller et al. 1976; Struckmeyer and Riker 1951).

According to Mullick (1977) periderms are non-specific responses thought to protect living tissues from the adverse effects of cell death. Although periderm formation appeared to be a primary factor retarding the growth of the fungus, defense reactions such as reaction zones might be specific interactions of *C. quercuum* f. sp. *fusiforme* with certain slash pine families, and thus a separate process. Likely, the ability of an individual family to produce firm boundaries may be under genetic control. Therefore, the degree of resistance in a slash pine family also may be related with the ability to induce periderm formation. Further, cell proliferation and accumulations of fatty and granular substances in the cell lumen of phloem cells are thought to be common responses of host cells to infection and seem to be related to the ability of cells to develop a periderm (Jeweil et al. 1980a). Similar granulation was found in loblolly pine infected by *C. quercuum* f. sp. *fusiforme* (Jackson and Parker 1958). The inward extent of the reaction zone buried in the xylem induced a compartmentalized condition associated with restricted expansion of *C. quercuum* f. sp. *fusiforme*, and restored cambium activity. Jewell and Speirs (1976) made similar observations in 2-year-old infections of resistant slash pine families inoculated with *C. quercuum* f. sp. *fusiforme*.

It is suggested that families expressing the SG symptom type have response reactions similar to the resistant expression expressed in the families with partial resistance. Similar responses have been shown in pathosystems with *Phytophthora cinnamomi* and *Banksia browni* (Smith et al. 1997), and Puccinia *striiformis* and *Triticum aestivum* (yellow rust) (Broers and Lopez 1996). In these pathosystems, anatomical barriers, such as reaction zones accompanied by adverse effects on haustoria and intercellular hyphae, induce plant responses associated with rate-reducing resistance, affecting or inhibiting sporulation. Spores do develop at infection sites lacking necrosis despite slow rates of fungal growth. These conclusions agree with other authors who suggested that resistance of slash pine families to fusiform rust could be a quantitative trait (Miller et al. 1976; Walkinshaw et al. 1990).

Major differences between SO and the other two symptom types were: (1) reaction zones were much smaller and less intensively stained than the reaction zones in the RO; (2) cells in the reaction zones were empty and often contained fatty-like substances, and hyphae and haustoria were often observed in the reaction zones; (3) hyphae were unusually multiseptate; and (4) much more colonization was observed in all sectioned regions than in RO and SG. Thus, it is suggested that this symptom may be indicative of a progressive reaction to the pathogen, which could depend on the fitness of the fungus or a quantitative expression of resistance.

It was concluded from the previous chapters that the slash pine families R1, R2, and R3 may have different mechanisms of resistance to *C. quercuum* f. sp. *fusiforme*. This hypothesis is based on the frequency of the different symptom types of galls expressed by the resistant families. For example, RO occurred more frequently in families with intermediate incidence (about 50%), such as family R3, and families expressing high incidence of PSG, such as families R1 and R2. Moreover, under field conditions these families were more stable than families expressing intermediate incidence under greenhouse conditions (Schmidt et al. 1999). Further, in Chapter 4, it was concluded that *C. quercuum* f. sp. *fusiforme* does not sporulate or sporulates at a very low frequency in families expressing SG.

This study provides the histological understanding of the defense mechanisms operating in slash pine families expressing resistant type symptoms (gall types) that have been used to predict field resistance. These families could be used in breeding programs and in research to gain a better understanding of the genetic mechanisms of resistance in slash pine families.

Conclusions

This study suggests that resistant slash pine families may have more than one type of defense mechanism active against *C. quercuum* f. sp. *fusiforme*. These mechanisms are reaction zone formations as the protective layer of parenchyma cells limiting fungal growth and extension. However, the size, rate of development, and intensity of the reaction zones were the conspicuous histological differences among the three symptom types.

Rough galls (RO) show a variety of incompatible and compatible reaction in the same tree. For example, the constricted areas were often associated with bark lesions, resembling those of SYMNOS; while the swollen regions showed characteristics similar to that of typical fusiform rust galls. The extent of response, and pathogen development in and adjoining the reaction zone, supports the hypothesis that the symptom-type RO appears to be intermediate between a susceptible and resistance reaction, and it is unclear if this trait expresses qualitative or quantitative resistance.

These results suggest that resistance of families expressing short galls (SG) is similar to partial resistance expressed in other rust pathosystems, such coffee leaf rust and poplar rust on both types of resistance.

Further, it is also hypothesized that these resistant gall types appear to be associated with quantitative response of the host to the pathogen. The observed resistance was associated with reduced fungal growth, limited haustorium formation, and appearance of necrotic areas which seem to be more pronounced with increasing levels of resistance.

CHAPTER 6
CONCLUSIONS

The major objective of this research was to determine if the differential fusiform rust incidence incited by *C. quercuum* f. sp. *fusiforme* on resistant slash pine families among year and locations observed in the Rust Virulence field trials were caused by pathogenic variability rather than by climate and phenology, or other confounding variables present in the field. First, rust incidence between years was evaluated using eight diverse pathogen isolates from two time periods (year of gall initiation) and four locations in the Rust Virulence field plantings (Chapter 2). Then rust incidence among location- and family-isolates was evaluated using 16 single-gall isolates collected at the same locations from specific families in the Rust Virulence plantings (Chapter 3). Both studies were conducted under greenhouse conditions at the Resistance Screening Center, Asheville, NC, using a concentrated basidiospore suspension technique. Secondary objectives of this research were to observe the sporulation pattern on families expressing high frequencies of short or large galls (Chapter 4), and to study the patterns of colonization of the pathogen in the symptom types of rough, short and smooth galls (Chapter 5).

Two conclusions could be drawn from the temporal (year) and spacial (geographic) variation studies: (1) families reacted differentially to inocula collected in different years, indicating that temporal variability occurred within certain families (Chapter 2); and (2) families reacted differentially to the single gall isolates collected from different locations and from specific families (Chapter 3). The results presented from these artificial inoculations, under controlled greenhouse conditions with mixed-gall isolates or with single-gall isolates, where only inocula were a significant source of variability, suggest that the differential family responses were primarily the result of pathogenic variability in both field data and greenhouse inoculations.

Regardless of family, trees characterized by short galls had smaller lesions size had reduced frequency of sporulating galls forming spermatia or aeciospores, and fewer numbers of spores produced per gall surface area, than trees characterized by large galls (Chapter 4). Comparisons in sporulation patterns between the susceptible and the resistant families indicated that the pathogen always sporulated more in the susceptible family than in the resistant families. Among the resistant families, the number of spores produced per gall surface area varied. From a practical epidemiological standpoint, the qualitative response of seedlings with limited fungal sporulation, especially those with less aecial sporulation than spermatia, would contribute to limited inoculum in the field, therefore possibly minimizing the frequency of pathotypes capable of infecting the improved trees. It was hypothesized that families expressing high frequencies of short galls may express a resistant type similar to that of partial resistance (quantitative resistance) found in other pathosystems. Examples include cereal rusts and coffee leaf rust pathosystems.

The histological study (Chapter 5) suggested that slash pine families resistant to fusiform rust may have more than one type of defense mechanism operative against *C. quercuum* f. sp. *fusiforme*. The primary resistance mechanism observed in this study included reaction zone formation as the protective layer of parenchyma cells limiting fungal growth and extension. However, the size, rate of development, and intensity of the reaction zone were the conspicuous histological differences among the three gall types. Moreover, this study provides histological understanding of the defense mechanisms that operate in slash pine families expressing these resistant symptom types, which have been used in predicting the relative field performance of slash pine families.

Durable disease resistance can be achieved either through quantitative (non-race-specific) resistance or by combining quantitative and qualitative (race-specific) resistance. Such resistant families could be useful in breeding programs and in a better understanding of the genetic mechanisms of resistance in slash pine. However, research is needed on the biology, ecology and genetics of the host and pathogen interaction. Investigations on the nature of resistance mechanisms, for example qualitative versus quantitative resistance, and investigations of the genetic structural and biochemical characteristics of fusiform rust galls need to be explored. Information gained can be used to explore other mechanisms of defense, such as gene activation in pines, as well as to provide genes that may prove useful in genetic engineering programs. A second area of interest is to better understand the biology of the fungus, such as fungal dikariotization, and influence of oak species on pathogenic variability. This research would provide a broader picture of the genetic variability present in the pathogen population. Efforts also should be made to monitor the frequency of pathotypes in the field. With this additional knowledge, a stable test plan could be established so that durable resistance would be achieved.

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